An Introduction into Patents

with PER.C6 example

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Colophon

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The source can be found on Github.

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Chapter 1

Introduction

1.1 To the audience

This reader presents an introduction on the use of know-how and intellectual properties (IP) and its benefits for students in science, engineering, medical and business courses. The basic concepts and definitions of IP will be treated and also their use and purpose will be described.

The different topics of IP are explained with an example relevant to your background.

Those interested will find additional information in the appendix appendix B by following the links.

1.2 Every day IP

Chances are that you are using products or services appropriated by a variety of intellectual property rights (IPR) on a daily basis, eg. brands, designs, patents, copyrights.

Many of the products that you will buy or use daily are from a certain brand. Such a brand makes you recognize the product and the manufacturer. For example the brand Coca-Cola for cola. On the other hand manufacturers and organisations use their brands to market their products and services.

Next to brands, organisations have their tradenames registered at the Chamber of Commerce.

The book you are reading or the music you are listening to are works made by an author or musician. These makers would like to be rewarded for the efforts put into the making of their work. You are therefore not allowed to copy this work without their permission since it is copyrighted.

In the development and production of bicycles and cars there are many proprietary technologies. Manufacturers of these product would like to earn back their investments in research and development by using patents.

When you are already developing products yourself now or in the future and when involved as entrepreneur or manager you will have to work with different kinds of IP. As a student it is therefore useful to acquire sufficient knowledge of IP for your future career. Even during studies you it can be worthwhile to use them for many reasons, for example for design assignments.

1.3 Why do IP rights exist?

Several hundred years ago the use of intellectual property rights was hardly known. At the beginning of the book printing technology it became possible to copy and disseminate works of literature far more easily. From that moment authors and publishers started to feel the need to appropriate the rights for the production and distribution of these works. With new technologies during the Industrial Revolution mass production in large quantities became feasible for products and devices. This gave rise to a growing interest by manufacturing companies to appropriate trademarks, logos and patents for their products and inventions.

The modern patent in Venice

During the fifteenth century, Venice was a rich and flourishing city. One of the reasons for this prosperity was the stained glass produced on the island of Murano.

This was a rare and expensive product that became an important economical asset for the city.

However, the formula for making coloured glass was known only to a few people: the glassmakers of Murano.

The Senate of Venice began to worry about the possibility that the glassmakers might die or flee to other countries, thus losing this precious secret.

To avoid such hypothesis, Venice offered the glassmakers to train some apprentices sent by the city. However, the glassmakers refused because accepting the offer would have meant that they loose their monopoly and create potential competitors.

Understanding Murano's concern, Venice offered, in exchange for the secret, an exclusive right for a limited time to guarantee

the glassmakers monopoly. The document granting this right was called a “patent”, from the Latin verb “patere”, meaning to make known.

Thanks to this, the craftsmen accepted the offer and Venice managed to keep the secret, so that we can still enjoy the beautiful coloured glass of Murano today.

In 1474, Venice published the first patent statue in history, to regulate the matter. See figure 1.1.

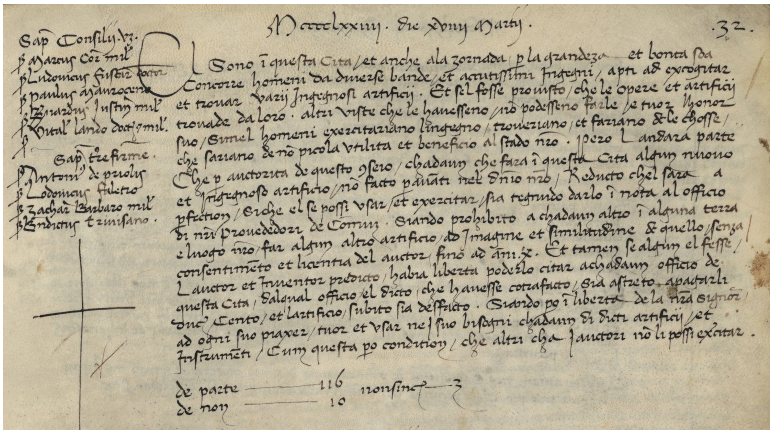

Figure 1.1: The Venetian Patent Statute, enacted by the Senate of Venice in 1474, is widely accepted to be the basis for the earliest patent system in the world.

The general concept behind the use of intellectual property rights is that the creator or manufacturer can apply for a temporary exclusive right hence appropriating their (often intangible) assets and stopping competitors. By doing so the IP owner acquires the possibility to exploit the production of these assets which are otherwise easily copied or manufactured by competitors. So, on the one hand intellectual property rights incentivize persons and innovators who invested both time and money to develop a new product. While on the other hand competitors cannot copy the product and sell it at a cheaper prices without making such investments.

Consumers of those products which have been appropriated with intellectual property rights may have to pay a higher price. Without these intellectual property rights competitors would have been able to sell the products at a lower price. For society at large the introduction of IPR is not only to have all products available at the lowest prices, but to have access to new products and innovations. While using IPR innovative companies are temporarily in a position to charge higher prices thus enabling a return on (earlier made) investments. This is shown in figure 1.2.

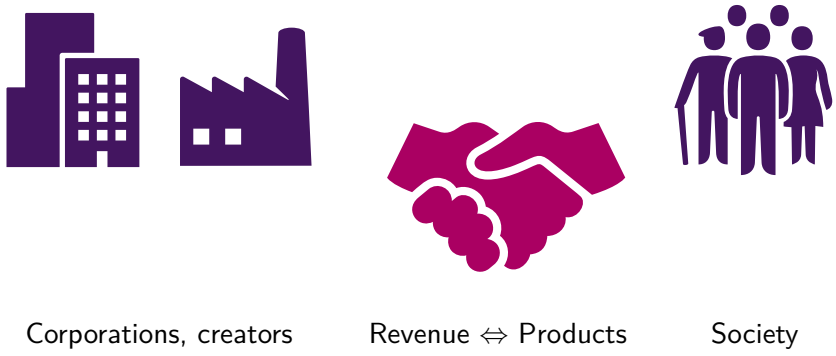

Figure 1.2: Use for business and society

1.4 Well known IP

Companies, entrepreneurs, authors, engineers, developers, scientists and inventors can use a variety of IPRs like copyrights, trademarks, patents, trade names, logos, designs, databases, plant breeders, integrated circuit layout and trade secrets.

Some of the well known IP rights are:

Copyright Will give the creator (author) at the end of the creation automatically global protection for original works like text, music and images. Copyrights limit free distribution of the work.

Trademarks After registration, the trademark owner receives the exclusive right to use the trademark for certain goods and services. A trademark right can be used to take action against competitors who want to exploit the same or similar trademark in the same market.

Patents After the application, registration and examination of a patent, others can be excluded from the commercial exploitation of the patented invention.

Tradenames Trade and company names are used to make a company known to customers in the market and ensure a reputation and thus customer loyalty. Another company may not cause confusion with its trade name by using a trade name that is too similar to a previously registered trade name.

Designs After registration, the design holder receives the exclusive right to use the design. A design right can be used to take legal action against competitors who wish to exploit a similar design.

1.5 Frequently used IP for innovations

This document will not describe the legal aspects of IP. See the links to several articles of different laws in appendix D. We will describe how to use IP, and more specifically for innovations. An overview of the importance of the different IP rights for innovations can be seen in the following table.

Table 1.1: Effectiveness of appropriability mechanisms for product innovations; % product innovations for which deemed effective.

Sector	n	Secrecy	Patents	Other IPRs	Lead time	Complementary sales services	Complementary manufacturing
Food	89	59	18	21	53	40	51
Petroleum	15	62	33	6	49	40	36
Basic chemicals	35	48	39	12	38	46	45
Drugs	49	54	50	21	50	33	49
Machinery tools	10	62	36	9	61	43	35
Computers	25	44	41	27	61	40	38
Electrical equipment	22	39	35	15	33	32	32
Semiconductors	18	60	27	23	53	42	48
Medical equipment	67	51	55	29	58	52	49
Autoparts	30	51	44	16	64	45	53
All	1118	51	35	21	53	43	46

From: Scotchmer [Sco04] Table 9.1, page 260.

Source: Cohen, Nelson, and Walsh [CNW00], table 1. Note: Each number is a mean response, representing the percentage of product innovations in the row category for which the type of protection in the column is deemed "effective". The response categories are <10%, 10%–40%, 41%–60%, 61%–90%, >90%.

In general we can see that secrecy (including what we call know-how) is one

of the most frequently used appropriability mechanisms. At the same time patents are important in the sectors drugs and medical equipment.

Other IPRs (for example trademarks or designs) are less frequently used for innovations, but are of course very important for sales and marketing.

1.6 An example

In this section we introduce the example which will be elaborated in next chapters.

Here, the main example is the research into and subsequent development and commercialisation of the PER.C6 technology. This research is about a vector to produce adenovirusses and a cell line to encapsidate the virus. In the years between 1992 and 1996 scientific research at Leiden University lead to the development of this technology for future use in gene therapy. Already in 1984, prof. Dinko Valerio started scientific experiments into this technology as part of his work as PhD student at Leiden University. He was one of the pioneers in the Netherlands and after his return from the USA he incorporated Introgene plc. together with prof. van Bekkum. Next, the company started a series of research cooperation projects with prof. Van der Eb and dr. Hoeben at Leiden University focussing on the development of cell lines with improved features. During the experiments they showed that several virusses, eg. adenovirusses, can be used for genetherapy as well as for a production platform of multiple vaccines ands medicines. Formerly it was known that adenovirusses can be used for gene therapy however their research enables certain improvements. PhD student Frits Fallaux and dr. Bram Bout were involved as scientists and inventors in a number of those experiments.

The invention concerns the production of adenovirusses which can be used as vectors and a cell line to package the virus. The idea behind the invention is to solve problems of existing production processes of adenovirusses as a vector. See figure 1.3 for the functioning of the Adenovirus as a vector. In 1996 Leiden University applied for a patent (WO 97/00326) for this invention.

Various studies from the World Health Organisation WHO show that some four percent of the global population suffer from gene related diseases. As such, the PER.C6 technology developed at Leiden can offer an interesting possibility to help solving this serious unmet societal need and health related problem. On the other hand it is widely acknowledged that the pathway from research, development, clinical trials, market authorisation for a therapy or the manufacturing of novel vaccines and medicines is extremely expensive and seldomly executed by one company only. Therefore, in last

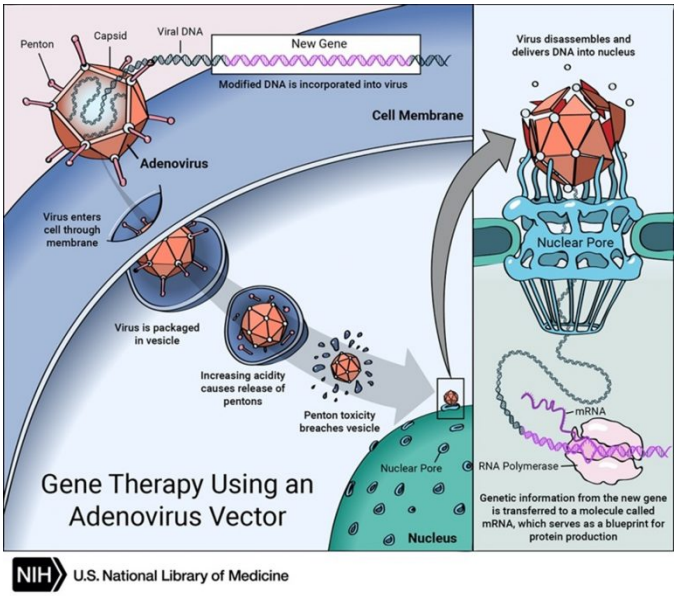

Figure 1.3: Gene therapy using an adenovirus vector

decades it has been become common practice that scientists at universities and medical centres cooperate with pharmaceutical and spinoff companies for the development of such medical services and materials. The example of Introgene plc. can be considered as an interesting case by itself. Next we see that IP and clear instructions on IP ownership and management often play an important role in the process and development of novel drugs, vaccines and therapies. And in particular patents and trademarks.

The most important patent application for the PER.C6 technology can be found and read as section E.1. This patent application will be used for further explanation about the use of patents in chapter 3.

The idea to use adenovirusses for gene therapy has been published earlier. But the new celline of the research group of Valerion has several advantages compared with existing technologies:

- The chance that competent virusses for homologous recombination will grow is reduced considerably by not having overlapping sequences between vector and adenovirus sequence in the cell line,
- There can be 38 kb pairs of strange DNA induced into adenovirusses. Previously it was limited to 7 kb.
- The use of a adenovirus is not limited to one host by host range mutation,
- The expression of the toxic adenovirus protein E2 is controlled by

  (1) mutation which restricts that proteïne E2 to bind to DNA within

certain temperatures only
(2) induced promoter

- expression of viral proteins in transduced cels, avoiding inflammations in cells.
- Viral replication proteins E1A and E1B only appear in packaging cells PER.C6 (PGK-E1-Retinoblasts) and not in the adenovirus construct.
- The adenovirus only consists of a minimal set of parts: encapsualtion signal, inverted terminal repeat, mutant E2 protein and target DNA.

Chapter 2

Know-how and trade secrets

2.1 Introduction

Know-how and trade secrets are important assets for companies and public research institutes.

Many entrepreneurs consider know-how as one of the most valuable assets of their company. Hence and although know-how is not a IP right as such we will go into know-how in this chapter.

2.2 What is know-how?

> Know-how is defined by certain knowledge and skill set obtained by a limited number of specific persons involved in manufacturing, marketing and sales processes of an organisation. By its very nature know-how is not accessible freely or without certain limitations to third parties and persons.

General knowledge in textbooks available to everybody is not considered know-how. See for an example of this definition of know-how Nieuwenhoven Helbach, Huydecoper, and Nispen [NHN02] chapter 5 (in Dutch).

In this context, third parties can be defined as organisations or persons who do not have access to certain know-how. In general very few persons within an organisation have access to specific know-how. Third parties and outsiders will always have to invest considerable time and resources to build up comparable know-how. As such, we conclude that know-how in an organisation is kept secret from third parties.

It is evident that persons must possess certain kills and knowledge in order to fulfill certain processes and tasks, for example the design and assembly

of a product, the draft of an algorithm, the acquisition and analysis of data. Therefore know-how consists of the combination of technical skills, the processing of information thereby using technical knowledge. Besides, non-technical knowledge like market data, marketing techniques, information about rules and regulations within a political context, data about relations and networks are also part of the know-how of organisations.

Investments in research and development contribute to the formation of valuable know-how, as well as working experience of and technical courses for personnel. In this case the acquisition and storage of information like technical data, equations, standards, specifications, processes, methods, recipes, drawings and their use by professional personnel.

2.3 Using know-how

Many corporations, public research institutes and multinationals have a division with IP specialists or in house council. They make sure that procedures, certain rules and codes of conduct concerning IP and know-how are in place and will be followed upon. Such procedures and conduct are often mentioned explicitly in labor contracts. An example of this is a non-disclosure clause.

But also at small and medium sized enterprises or startup companies without in house IP specialists or council it is important to implement internal procedures and codes of conduct to deal with IP and know-how. For those companies which supply parts, products or processes in a supply chain these procedures and codes of conduct are even more important. Without them such companies may run the risk that employees share too much essential know-how with customers or clients.

2.3.1 Using know-how by the company itself

The use of IP rights enable companies to have a positive return on investment in their research, development, marketing and manufacturing with a healthy commercial margin. On top of this, it is important to realise that the combined use of know-how and patents contribute to the successful introduction of technical innovations in the marketplace. In this process know-how of specialists is essential to deliver products and services to customers and clients. In the economic domain the concepts and use of know-how and patents show a striking number of resemblance. Both are a source of (technical) knowledge enabling the owner and user to use technical capacities and developments and thereby a head start or lead advantage which is not available to competitors. The owner of the know-how can exploit this

technological advantage in the marketplace, for example in certain manufacturing processes.

2.3.2 Using know-how by third parties

Many companies do not have manufacturing plants in all countries over the globe. In those countries where there is an outlet for their products or services but where they are not operational themselves in terms of manufacturing, marketing and sales it may be profitable to act as a licensor and work with license agreements. These license agreements are often struck for both patents as well as for know-how. The temporary, exclusive nature of patents provide either the patentee or the patent licensee protection against infringement by competitors. On the other hand, license agreements between the licensor and licensee determine the scope and field of use, geographical area, region or country, time frame in years and royalties or milestones to be paid.

2.4 Rules and regulations

Rules and regulations for know-how can be found in the EU directive 2016/943 and in the Dutch Act of Trade secret protection.

> This act rules the protection against unlawful public use of know-how and business information. This combination of know-how and business information is often defined as trade secrets.

According to the act and the directive a company or organisation must comply to certain conditions with regard to the information which:

a. is kept secret because it is not common knowledge or accessible by third parties,
b. has value in relationship with the trade or transactions of the company or organisation, and
c. is kept secret by the company or organisation by means of certain measures (for example a registration system and limited accessible for persons only on a need to know basis).

All in all it must be clear that know-how is a personalized asset. At the end of a labor contract the know-how does not automatically disappear (see figure 2.1). This situation raises the question if know-how can be claimed by the employer at all?

Figure 2.1: Know-how: There it is and there it goes.

Chapter 3

Patents

3.1 Introduction

With a patent you become the owner of your invention.

Thus a patent is property which you can use:

a. preventing others to use your invention, or
b. giving permission to others to use your invention.

The concept of property is defined under (inter) national law and regulations. This is also true for patents since patents are part of industrial property rights. Using a patent in a specific country will always depend on the framework of laws and legislation in that country.

Since the use of an invention is often not limited to a particular country only, it can be profitable to use it in other countries as well.

The world of inventions is therefore multinational or worldwide.

Since patents are used on a globally there are several international treaties for patents next to national patent laws. An introduction into the most important international treaties can be found in section 3.2.

Most relevant features of patents are elaborated in following sections.

From section 3.6, the contents of a patent will be described using the main example (see section 1.6).

3.2 Patent laws and treaties

Every country has its own patent law. In addition, there are often regional or international cooperations through treaties. An example of such a regional cooperation is the European Patent Convention. This European cooperation has ensured that the patent laws in the 38 member states are harmonised. There is also a global treaty for a central worldwide patent application through the World Intellectual Property Organization (WIPO) (193 member states).

- The Dutch Patent Act is determined in the Rijksoctrooiwet 1995 (ROW). The Netherlands Patent Office (Octrooicentrum Nederland) grants Dutch patents.
- The patent law for European patents is determined in the European Patent Convention (EPC). A European patent is granted by the European Patent Office (EPO). Next they are registered by the applicant in the countries of interest.
- The route a worldwide patent application is determined in the Patent Cooperation Treaty (PCT). However, no patent will be granted in this procedure. After this central application, the patent application is continued in the countries or regions of interest.

3.3 Patent rights

Patent law excludes others from commercially:

- making,
- using,
- selling, or
- stocking

the invention.

Such exclusivity lasts for a maximum period of 20 years after the filing date of the patent application.

The restrictions that a patent exerts are determined by the legislation of a country in question. These restrictions can therefore differ greatly from country to country. It should be noted that the Treaty of Paris (1883) guarantees a minimum harmonisation.

In Europe, a patent generally restricts the commercial making, use, sale and stocking of the invention, but it does allow to use the invention for one's own non-commercial use.

> So you can build a Ferrari for yourself, but don't sell it to your neighbour, because that would be a commercial act.

Under certain conditions, it is also permitted to use the invention for scientific and research purposes, without being able to be prosecuted for infringement.

For a precise description of the legal consequences of a patent in the Netherlands, see article 53 ROW (in Dutch).

The patent right can no longer be used if the patent holder, or someone else with the consent of the patent holder, has sold the patented product. You can then do whatever you want with the patented product. This is called exhaustion. This is described in article 53 paragraph 5 ROW.

3.4 Inventions

Most people have a general idea about inventions and inventors. For example, it is:

- a new development,
- often with a technical background and
- an improvement over existing technologies.

More formally, an invention is often described as a technical solution to a problem.

> However, an invention is not defined in patent law!

In patent law, the definition of an invention has been avoided by defining accurately what is not considered an invention. For example, theories and mathematical methods are not regarded as inventions hence they cannot be patented.

Furthermore, an invention must be industrially applicable. This requirement of industrial applicability separates patent law from the other intellectual property rights.

The requirements for novelty and inventive step ensure that certain technical developments and inventions are only considered to be patentable inventions, if their subject-matter is not already known by (or disclosed to) the public and is also not obvious.

For a more accurate description of the exceptions on patentability and the basic requirements, see article 52 EPC or article 33(1) PCT.

3.5 Requirements for a patent

There are many requirements that a patent must meet. In addition to formal requirements, there are substantive requirements. Formal requirements are necessary for the proper processing of the application. For example, it is necessary that the patent office can contact the applicant and that the application is written in the correct language.

To obtain a granted patent, the most important substantive requirements are that the invention is:

- new,
- inventive,
- must be sufficiently clear disclosed.

The invention must be new and inventive, otherwise the patent would not contribute to the general knowledge and improvement of technology. It must therefore also be described clearly enough.

3.5.1 Novelty

Novelty means that the invention has not been disclosed. All information that is publicly accessible to the person skilled in the art can be used to determine this. It is an objective criterion, whereby the person skilled in the art is supposed to know all state of the art.

For the assessment of novelty (and inventive step) all information before the filing date of the application is taken into consideration. This is the date of the first filing: 'first to file'.

Until recently, the United States had a different system: 'first to invent'. The moment when the inventor conceived the invention was the moment for the assessment of the requirements. Although fundamentally correct, this brings with it all sorts of problems of proof when conflicts arise. That is why in 2011 the United States also switched to the 'first to file' principle.

Documents with a later publication date than the filing date can not be detrimental to novelty, nor can they take away inventiveness.

So if not all features of the invention are already known, the invention is new:

> An invention shall be considered to be new if it does not form part of the state of the art (see also article 54 (1) EPC or article 33(2) PCT).

3.5.2 State of the art

The state of the art is accurately defined in the patent law:

> The state of the art shall be held to comprise everything made available to the public by means of a written or oral description, by use, or in any other way, before the date of filing of the patent application (see also article 54 (2) EPC or Rule 64 PCT).

This definition stipulates that all information that is publicly accessible in the world is regarded as state of the art. This also includes the documents in a small library in a Chinese mountain village. An important limitation is that the information must be *publicly* accessible. Documentation, such as technical drawings used in a company, is normally not publicly accessible (due to confidentiality). These documents can therefore not be used to assess novelty.

The filing date is an important date. Anything that has become available public after this date will not affect the patent application. If the same invention is applied for on different dates, the person who applied first has the right to the invention.

Each patent application is published 18 months after the first filing. Thereby it also becomes part of the state of the art.

3.5.3 Inventive step

Inventive step means that it is not obvious for the person skilled in the art to carry out the improvement or modification, for which protection is requested, in the particular solution:

> An invention shall be considered as involving an inventive step if, having regard to the state of the art, it is not obvious to a person skilled in the art (see also article 56 EPC or article 33(3) PCT).

In the practice of patent examination, this means that all claimed properties are known from a combination of two embodiments, described in one or two documents. The person skilled in the art is thereby also hinted to combine the features of the two embodiments.

or

If the only difference with a known embodiment is an alternative that is obvious to the person skilled in the art, which he knows on the basis of his general knowledge, then the invention is considered to lack an inventive step. For example: To attach something on a wall, a screw is a well-known alternative to a nail.

3.5.4 Clear and sufficiently disclosed

In a patent, the invention must be made public. This must be done in such a way that it can be performed by the person skilled in the art. It is therefore not possible to obtain a patent and keep your invention secret. See also article 83 and 84 EPC and article 5 PCT.

> A perpetuum mobile is therefore by definition not patentable.[1]

Features that are well known by the person skilled in the art do not need to be described. For example: It is not necessary to describe how something should be fastened, if it is clear to the person skilled in the art that it can be either welded or glued.

The person skilled in the art is defined in patent law as skilled in the field of the invention with broad professional knowledge. The skilled person only knows obvious solutions to problems, but cannot become inventive himself.

3.6 Contents patent application

A patent application consists of the following parts:

Description The description consists of an introduction and a section containing at least one complete embodiment of the invention. The introduction briefly describes what is known in the state of the art, what problem still exists in this known state of the art and a short description of the solution (the invention) to this problem.

Claims The claims define the scope of the patent protection. These claims are normally written as a set of claims. Usually there is a main claim and several dependent claims. The main claim therefore offers the broadest scope of protection. The dependent claims add further features and therefore have a smaller scope of protection than the main claim.

Figures The figures are there to clarify the invention.

The claims determine the scope and type of protection. The legal scope of protection of the patent is therefore determined by the claims. The claims are therefore written in a legal style.

[1] Why is a perpetuum mobile not sufficiently disclosed? Click for explanation.

For maximum protection, the invention is described as broadly as is possible in the claims. But if the invention is described too broadly, then the possibility increases, that it is deemed not new or not inventive.

3.7 Publication patent application

The patent application is published 18 months after the first filing. Figure 3.1 shows the front of the publication of the PER.C6 application. After the front page, the pages of the application as filed are published. The whole publication can be seen in section E.1.

This is the A publication (see the A1 code in the publication number WO 97/00326 A1). The A publication is the publication of the patent application. The next publication is the B publication. The B publication is the publication of the granted patent.

Bibliographic data are published on the first page of a patent document. The following data are the most interesting:

Title gives a very quick indication of the subject of the patent.

Abstract gives a short summary of the contents.

Figure next to the abstract is often a figure from the list of figures which is representing the invention.

Other data on the first page are more interesting to check for the legal aspects of the patent document:

Applicant is the one who has filed the application and the one who will normally have the patent rights.

Inventor is one person or are more persons who have made a significant contribution to the invention. In US patent law, the inventor is the one who has the rights to the patent. In other countries it is the applicant who has these rights.

Priority data is the date of the first patent application filed and for which a priority is claimed. The patent rights start from this date. In this case there are two European priority documents (EP 95201611 from June 15 and EP 9521728 from June 26 1995).

Filing date is the date this application was filed.

Designated states are all the countries that are requested for patent protection when this application was filed under the PCT. The PCT procedure is used to start a world wide patent application.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 5/10 // A61K 48/00

A1

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(54) Title: PACKAGING SYSTEMS FOR HUMAN RECOMBINANT ADENOVIRUS TO BE USED IN GENE THERAPY

(57) Abstract

The invention provides improved methods and products based on adenoviral materials which can advantageously be used in for instance gene therapy. In one aspect an adenoviral vector is provided which has no overlap with a suitable packaging cell line which is another aspect of invention. This combination excludes the possibility of homologous recombination, thereby excluding the possibility of the formation of replication competent adenovirus. In another aspect an adenovirus based helper construct which by its size is incapable of being encapsidated. This helper virus can be transferred into any suitable host cell making it a packaging cell. Further a number of useful mutations to adenoviral based materials and combinations of such mutations are disclosed, which all have in common the safety of the methods and the products, in particular avoiding the production of replication competent adenovirus and/or interference with the immune system. Further a method of intracellular amplification is provided.

Figure 3.1: Front page of WO 97/00326 A1

Publication date is the date this application was published and thereby known to the public. Before this date, the application was secret and not known to the public.

Also some administrative data are mentioned, so that the document can be easily identified:

Publication number is a unique number to identify a patent document. It also gives information on the type of document. The first letters are the country code. In this case WO, which stands for the PCT world wide application. Others are for example EP for the European procedure at the European Patent Office (EPO), NL for the Netherlands, US for the United States, DE for Germany, etc. There is also a kind code. In this case A1, which stands for application published with search report. When an application is granted, then often the B code is used.

Application number is the number the application gets when it is filed.

There are also classification codes published on the document. These codes are used for searching.

3.8 Claims

The claims determine the scope of protection of the patent. Usually there is a main claim with several dependent claims. The dependent claims define further features of the invention.

The function of the dependent claims is to have more specific claims in case the main claim does not hold up in the examination procedure or in court.

3.8.1 Claim of the PER.C6 example

The main claim of the life sciences and health application is as follows (WO 97/00236 A):

> A recombinant nucleic acid molecule based on or derived from an adenovirus having at least a functional encapsidating signal and at least one functional Inverted Terminal Repeat or a functional fragment or derivative thereof and having no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which it is transferred.

The language used in the claim is a lot more complicated than the language you might normally use to describe the invention. The invention can also be described as:

> The functional inverted repeats and incapsidated signals of adenovirus DNA which are necessary to replicate in cells.

One reason for this complicated language in claims is that the text is a legal text. The invention must be legally clearly described. This claim describes a functional group which is needed for the therapy. Some special words have been used like 'based on' or 'derived from' and 'derivative thereof' defining on the one hand the invention but not limiting it too much on the other hand.

3.8.2 Test of novelty

As mentioned earlier, a patent must be new. In the search report in the publication of the patent application in section E.1, other documents are shown as state of the art. The examiner uses the search report to assess novelty and inventive step of the patent application. Some older documents describe the features in the claims thereby destroying their novelty.

The following demonstrates how novelty can be assessed. It starts with breaking down the claim into separate features. It is then determined whether these features are collectively known in a prior art document. In this exercise the first patent document mentioned in the search report is used. This document with number WO 94/28152 can be found in section E.2.

Try to find the answer yourself before viewing the answer.

Features claim 1 of the patent application	**Where to be found in WO 94/28152 A?**
A recombinant nucleic acid molecule based on or derived from an adenovirus	Click for answer.
having at least a functional encapsidating signal	Click for answer.

and at least one functional Inverted Terminal Repeat or a functional fragment or derivative thereof	Click for answer.

having no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which it is transferred	Click for answer.

When reading the contents of the table it is clear that the main claim cannot be granted due to lack of novelty, because all features are already known from document WO 94/28152.

Besides, the examiner makes an objection regarding that the original claim misses an essential feature. The claim describes a recombinant adenovirus having certain adaptions in order to prevent the occurence of competent adenovirusses. But for the prodcution of the adenovirusses a specific cell line is used, therefore this should be included into the claim.

3.9 Patent application procedures

Patents can be applied for in different countries, but also regionally. In Europe, a European patent can be applied for at the European Patent Office (EPO). A worldwide patent application can be applied for via the PCT procedure at the WIPO.

These different patent application procedures have great similarities, but they are not the same. Therefore, the different procedures are briefly described below. Finally, the procedures chosen in the example are described.

3.9.1 EP patent application

The patent application procedure for a European patent (EP) will be discussed first. This procedure is similar to the patent application procedures used in many countries. Figure 3.2 shows an overview of the EP procedure.

The application starts with the filing of the application at the patent office. The first substantive response to the request is a search report. The most relevant state of the art is mentioned in the search report. The state of the art mentioned in the search report is used in the assessment of the

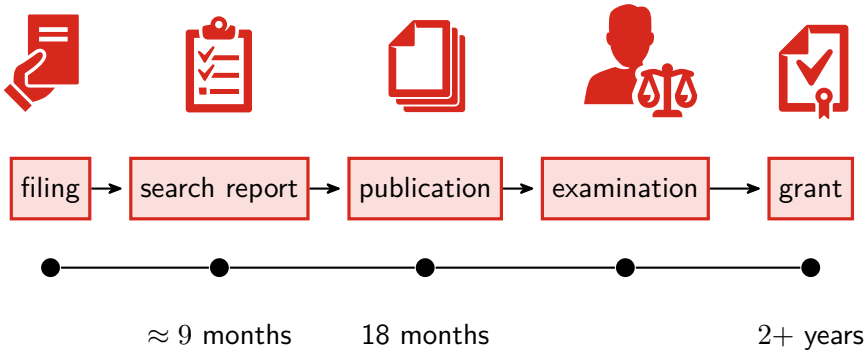

Figure 3.2: EP procedure

requirements for a patent. This assessment of the requirements takes place during the examination. In addition to the search report, a written opinion is delivered with the search report. Possible objections to the grant of the patent are noted in the written opinion. Not being new or not having an inventive step are the most well-known objections.

The application will be published 18 months after the first filing date. Up to the publication date, the application is secret. From the moment of publication, the invention is known all over the world.

Before the patent is granted, first an assessment is made whether the application meets all the requirements. If not all requirements are met, a communication will be written by the examiner and sent to the applicant. This communication states the objections against the grant and that the application can thus not be granted. The applicant has the possibility of overcoming these objections, for example, by amending the claims. This round of objections and amendments can take place several times. At the end of the procedure an oral hearing may also be held to come to a decision.

If there are no objections, the application will be granted. There is also the possibility that the application will be refused if the objections are not overcome.

After the grant, the patent must be validated at the national patent offices in the desired countries in Europe. The European patent then becomes a bundle of national patents.

3.9.2 NL patent application

The Dutch procedure for a patent is simpler than, for example, the European procedure. The Dutch procedure is shown in figure 3.3. A similar procedure is also used in other countries, such as Belgium.

Figure 3.3: NL procedure

The big difference with, for example, the EP procedure is that there is no examination. The patents are granted automatically together with the publication. Also patents that do not meet the requirements are automatically granted. The information from the search report and the accompanying written opinion must then be used to estimate the extent to which the patent holder can exercise his patent rights. A possible lawsuit will clarify these patent rights.

3.9.3 PCT patent application

The PCT (Patent Cooperation Treaty) procedure, for the worldwide application of a patent, is shown schematically in Figure 3.4. The single central application for the most relevant countries in the world is the advantage of the PCT procedure over national or regional procedures.

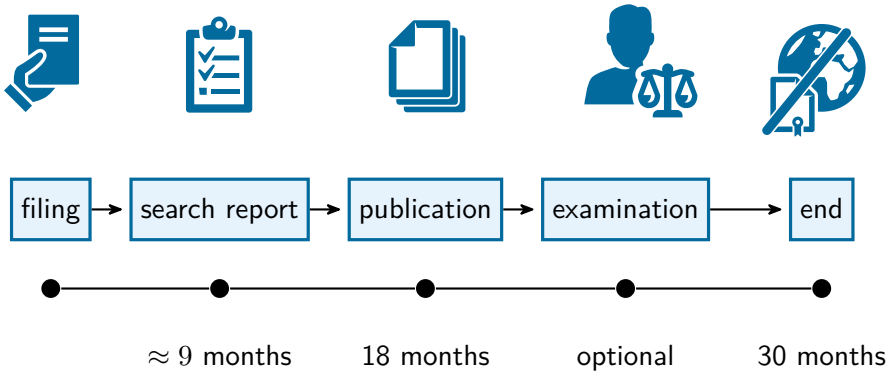

Figure 3.4: PCT procedure

However, there are 2 characteristics that form an important difference to the other procedures:

1. The PCT procedure ends after 30 months. At that moment no patent has been granted.
2. The examination is optional.

The procedure to obtain a patent must be continued in regional or national proceedings. So the PCT procedure is only the beginning of the patent procedure. The optional examination is therefore not a decision to grant or refuse the patent, but an opinion on patentability.

The postponement of the choice of the desired countries and therefore also a postponement of costs is a reason why often the PCT procedure is chosen. Furthermore, the costs of a search report happen only once, because the search report from the PCT phase is used in the later national or regional examination. Otherwise, if parallel applications were made in different countries, these costs would have to be incurred in all the selected countries.

The PCT procedure is therefore of interest if patent rights are expected to be desired in several countries in different regions.

3.9.4 Priority year

It is usually only possible to assess whether continuing the application is useful after receipt of the search report. That is why most countries have the rule that the priority of a previous application from another (or the same) country can be used for 1 year. The applicant then has one year to determine in which countries a patent is also wanted. The later application will then receive the priority date of the earlier application. It is then as if the later application was filed on the date of the earlier application (see also article 87 – 89 EPC or article 8 PCT).

This priority right can also be used for regional procedures such as the EP procedure or for the PCT procedure. It is therefore possible to start with the patent application in one country and then go to the worldwide PCT procedure within 1 year. You then have the opportunity to estimate the usefulness of the patent application before larger costs have to be incurred.

3.9.5 Procedure of the PER.C6 patent

Figure 3.5 shows an overview of the procedure from the application of this invention up to the grant of the patent.

First two EP applications were filed. These applications were withdrawn before publication.

Within a period of one year after these first applications, the patent application was submitted via the PCT procedure on June 14, 1996. This

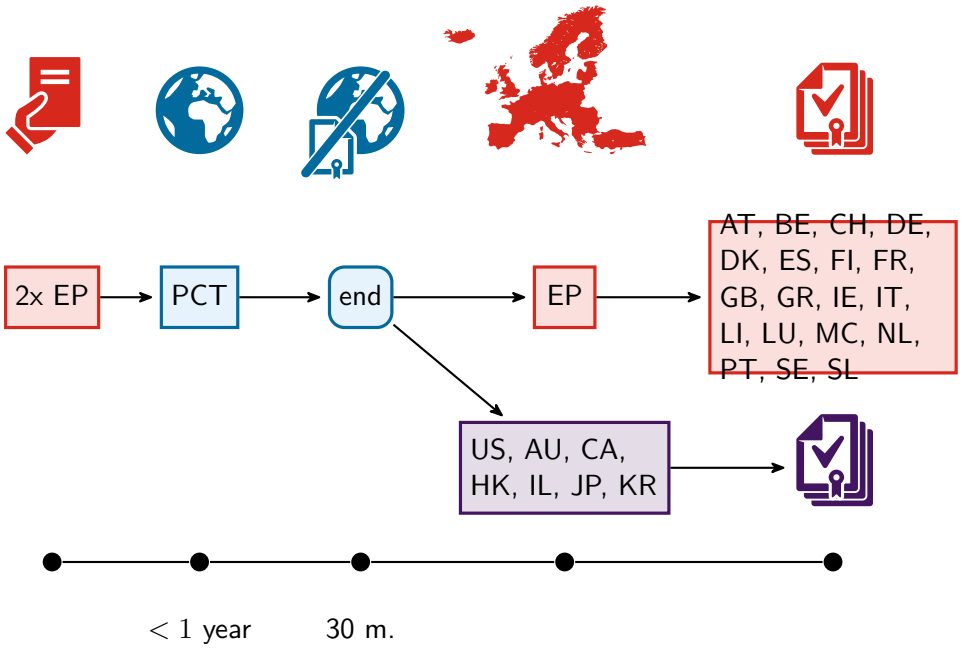

Figure 3.5: Procedure of the PER.C6 patent

application used the priority of the two EP applications. In addition to a short summary, this application also contains a more general description, together with scientific articles and results of underlying research.

An application will be published 18 months after filing and can be continued within the priority year through various application procedures. The PCT procedure ends after 30 months.

It has been decided to continue after the PCT phase .

Next it was decided to continue after the PCT phase through various national patent application procedures and the EP application procedure. Through the EP procedure patents have been granted and registered in 19 European countries: Austria (AT), Belgium (BE), Switzerland (CH), Germany (DE), Denmark (DK), Estonia (ES), Finland (FI), France (FR), United Kingdom (GB), Greece (GR), Ireland (IE), Italy (IT), Liechtenstein (LI), Luxembourg (LU), Monaco (MC), Netherlands (NL), Portugal (PT), Sweden (SE) en Slovenia (SL). Via additional procedures the patent has been granted and registered in Australia (AU), Canada (CA), Hong Kong (HK), Israel (IL), Japan (JP), Korea (KR) and the United States (US).

Although we cannot be certain it seems obvious that the market potential for this PER.C6 technology played an important role in the choice to apply for patent protection in those countries.

3.9.6 Granted PER.C6 patent

In the international search report for the patent application of the PER.C6 technology it was clear that three documents were identified that take away the novelty. During examination the originally filed claims have been amended. The granted main claim reads as follows (added text is in italics):

> *A pakaging system comprising*: a recombinant nucleic acid molecule based on or derived from an adenovirus, *said nucleic acid molecule* having at least a functional encapsidating signal and at least one functional Inverted Terminal Repeat or a functional derivative thereof, *and having a packaging cell said recombinant nucleic acid and said packaging cell together comprising all elements which are necessary to generate a a recombinant adenoviral particle comprising said recombinat nucleic acid molecule wherein said recombinant nucleic acid molecule has* no overlapping sequences which allow for homologous recombination leading to replication competent virus in *said packaging cell.*

Several features have been added to the main claim. With these additional features, the claimed PER.C6 technology is deemed to be clearly claimed.

You can see that the scope of protection as described in the claim set of the granted patent is reduced as compared with the patent application. This also indicates the importance of the dependent claims and of a sufficiently detailed and complete description. The claims can then be modified with additional features mentioned in the dependent claims or the description. With these additional features it is possible to overcome the objections to the granting of the patent. These features must already have been described in the patent application, as they can not be added later after the original filing of the application.

3.9.7 Patent family

You have seen from the example that a first patent application resulted in several equally granted patents in the different countries. These patent applications and granted patents have practically the same content. However, they are all published separately.

Most of these publications are included in the patent databases. However, when you are searching, you don't want to see every publication with the same content separately. That's not helpful. If you have seen one, you also know the content of the other publications.

In the patent databases, the publications are therefore grouped by family. A family of patents is therefore a collection of patent applications and patents that have the same content. The grouping is done automatically, using the relationship with the first filed application (the priority document) to group the documents. However, this may sometimes not be correct if a non-standard procedure has been followed.

3.10 After grant of the patent

It is only after the granting of the patent that it is clear what the exact scope of protection of the patent is. That is why the patent can only really be used to stop others using the invention once the patent has been granted. However, the work on the patent and also the costs and even risks are not over yet.

The following activities are still required:

1. You must discover potential infringement of your patent yourself. So you have to pay close attention to which competitor may be infringing.

2. You must also organize the stopping of a possible infringement yourself. Warn the potential infringer first and perhaps eventually even file a lawsuit. A lawsuit is not cheap. This will have to be taken into account when deciding on the strategy to be followed.

3. Even if your patent has been granted, you can still lose it. In the EP procedure, an opposition procedure is still possible within 9 months after the grant. During an opposition procedure, third parties can object to the granted patent. In that case, the patent may still be rejected. It is also possible that the patent needs to be modified. This is comparable to the examination of the patent application.

   The patent can also be attacked later through the courts by third parties. Also then is it possible that the patent will be declared invalid. This step is usually taken by third parties if they are accused of infringement.

4. To ensure that patent rights do not continue to exist for an unnecessarily long time, an annual maintenance fee must be paid. If payment is not made, the patent expires. If the patent does not have enough economic value, it is probably better not to maintain it any longer.

It is clear from the foregoing that the publications in the patent databases do not provide information about the status of a patent. This status must be looked up in the patent registers. Each country has its own patent register

to administer this status. Some links to these registers can be found in section B.4.

Chapter 4

Using IP to make money with technical innovations

4.1 Introduction

In this chapter we will discuss the topic of strategic management and use of industrial property rights in companies. Copyrights do not belong to the industrial property rights, but they deserve a specific place in companies.

Here it is also important to distinguish ideas from inventions and innovations as they are often used throughout or amongst one another. We presented a working definition of inventions in section 3.4. While some ideas about new products and services may lead to new research and development and further product development and hence towards inventions, most of them will not be used in the process of innovation management. As such those ideas will not be translated into inventions incorporated into to valuable innovations in certain sectors of industry. Because, on the other hand an innovation is most often regarded as a new and tangible product or service which can be bought by customers in the market place thus creating economic growth.

In the next section we describe a number of common steps in a company's innovation process as the basis for the use of IP. In the following sections the use and exploitation of IP rights is discussed in the various steps throughout the innovation process.

4.2 Innovation process

Often innovation is a time and resources consuming process going through various phases from first idea, prototyping, validation to market entry of an novel product or process. Throughout that innovation process information

about IP can be used in multiple ways. In figure 4.1 this innovation process is schematically pictured.

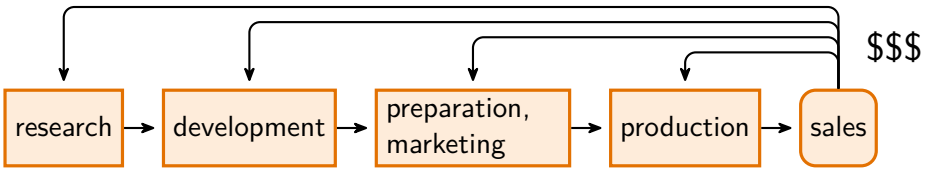

Figure 4.1: Proces from research to sale of product

Many companies start their innovation process by assigning market intelligence to one or more specialists. A state of the art research and necessary steps for product development may require significant time and resources depending on the sector of industry. For long term projects a company can decide to cooperate with a university for example for research ends working with scientists and PhD students. The goal of this phase in product development can be to ascertain proof of concept and bringing an idea for a novel product to the next stage.

At the development stage the product will be shaped towards the final version, although the manufacturing process at full scale is not yet determined. Since experts and engineers from various disciplines are involved in this stage, it can be time consuming and expensive.

Next, decisions about the output level of production and the layout of the factory have to be made during the production preparation phase before the start of a manufacturing process. Costs will usually depend on both the final product and sector of industry. For example building a construction plant for new cars can require initial investments of billions of euros.

Although marketing and sales do not seem a logical next step in an innovation process, they are of key importance. A successful market entry of new products will depend on sales to customers, thereby assuring that all investments and expenditures made earlier (like research and development, production engineering and marketing) will be earned back.

> Only the sales of the product generate revenues!

All steps in the innovation process prior to the stage of sales require adequate funding and investments. Those initial investments can be substantial while the return on these investments will be realised through sales. Using IP enables companies to create large enough margins when selling their innovative products to earn back those initial investments. Thus while IP contribute to the return on investment of companies, they can incentivize the market launch of their innovations at the same time. Conversely, intellectual properties only have value if a product is brought to market.

4.3 Using IP information for decision making throughout the innovation process

Using information from available intellectual properties in a timely manner is useful to avoid potential issues after market introduction of the product or to reduce certain costs throughout the innovation process.

In figure 4.2 the type of information that can be used and the moment of use is displayed.

We distinguish two kinds of analysis to retrieve and analyse such information;

1. patent landscape analysis

    a. Technical information about known solutions,
    b. Appropriated technical solutions with potential legal effect to take into account,
    c. A market analysis with names of competitors or potential partners

2. Freedom to Operate (FTO) analysis

    Information with potential infringement and risks assessment.

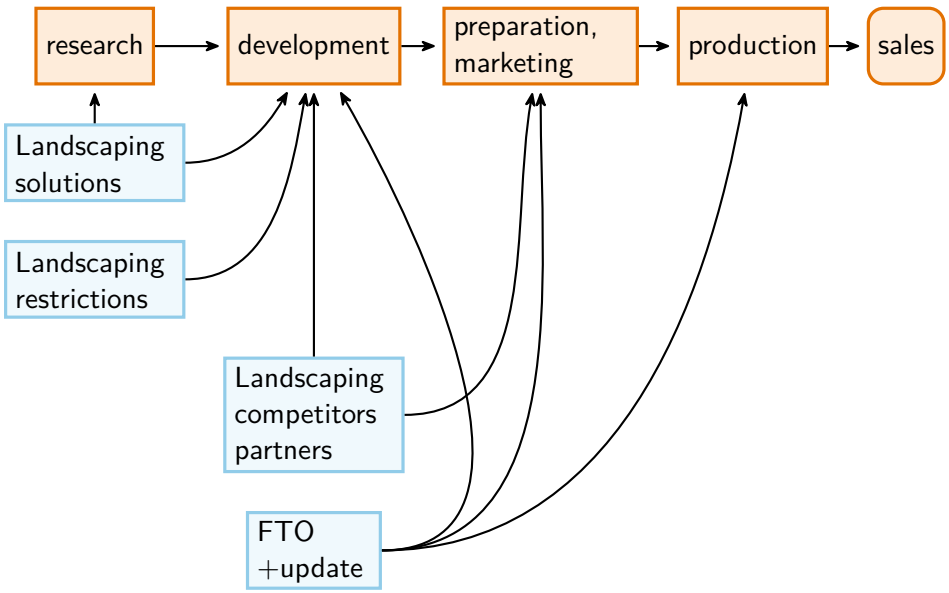

Figure 4.2: IP information in the process for a new product

4.3.1 Patent landscape analysis

In a global market companies and research organisations are surrounded by competitors and other actors. Using a patent landscape analysis one will acquire more information about them and about their technologies.

In a patent landscape analysis data can be analysed in three ways. Those three ways will generate useful data enabling easier decision making throughout different stages of the innovation process.

A. It is useful to create an overview of known technologies in order to be able to determine which problems and solutions need to be further analysed and developed within your organisation. For this analysis (technical) persons with knowledge of the subject-matter are necessary.

B. Prior to the decision to start developing a new product it is useful to study interesting technologies described by patents and pending patents. Search for possible technical solutions that may come close to the research and development of the organisation. Both technical and (legal) patent knowledge are required for these analyses. Analysing these data from a legal point of view may restrain your willingness to start a new innovation process. However, following decisions will depend on the business strategy of the organisation. Assuming that useful data have been retrieved and analysed one can decide to avoid potential litigation or infringement by redirecting the scope of research and development. A different strategy will be to license in the patents or start working as a partner of the patentee. These strategies will be further elaborated in next sections.

C. In addition to the technical and legal information from a patent landscape analysis, you can also obtain useful data for further market research. You can use this information to discover interesting countries, markets and possible partners for the sale of new products. It is also possible to analyze interesting markets in which you do not want or cannot be active yourself, but can become active through for example a partner.

4.3.2 Freedom to Operate (FTO) analysis

If the product reaches its final appearance at the end of the development phase, it could be useful to make an analysis about the risks to potential infringement of patents of third parties. An infringement of patents of third parties by may seriously hinder or even stop market introduction of a novel product or device. Such a risk assessment is called a Freedom to Operate analysis.

Throughout the patent landscape analysis one has analysed a first indication of potential infringement. But only when the the product is sufficiently specified and defined, an FTO analysis will be able to give sufficient certainty

of the risks. Until the moment that your product will become part of the state-of-the-art for example through sales, a publication or a patent, it is still possible that others will get IP rights that will hinder sales of the products. Therefore it is useful to update the FTO analysis.

An FTO analysis requires both technical knowledge and legal IP expertise. Also knowledge about legal and financial risks is required. Due to that multidisciplinary character of such an FTO analysis costs are high. Therefore scope and nature of an FTO analysis better be aligned with the risks and business strategy of the company.

4.4 Strategic IP use

For companies it is important to determine which sort of IP rights are needed for launching successful innovations. Bigger companies and established firms have their own IP division with an IP strategy in place. In line with their strategy they usually start applying for a diversity of IP rights during the various stages of their innovation process. More in depth information about commonly used IP appropriation regimes by economic sector, products and process innovations can be found Cohen, Nelson, and Walsh [CNW00] and Scotchmer [Sco04], chapter 9.

We know that IP can enable companies to create enough margins once they sell their products to have a return on their investments thus incentivizing innovations. As IP proprietor the innovator may decide to stop competitors to bring the same product or process at the market price at lower costs or prices. Such mechanism is called a defensive IP strategy and is generally used by companies in the pharma sector. Economic literature about such a price mechanism enabled by product or process patents is described by Greenhalgh and Rogers [GR10] in chapters 1 and 2.

Figure 4.3 describes which kind of IP can be relevant in certain stages of the innovation process.

During research and development leading to technical innovations patents often are used. When publishing articles about scientific results at universities copyrights are important. Depending on the sector of industry in which a company is operational designs becomes relevant at the stage when the product will have a clearly defined outer shape and the shape needs to be easily recognizable by customers.

Brands are important for the marketing of products and services. In the interest of marketing designs can be used as well.

Know-how (secrets) about certain features in a manufacturing technology process, for example the use of parts are regarded as yet another intellectual property. If a company has a more offensive IP strategy patents can be used

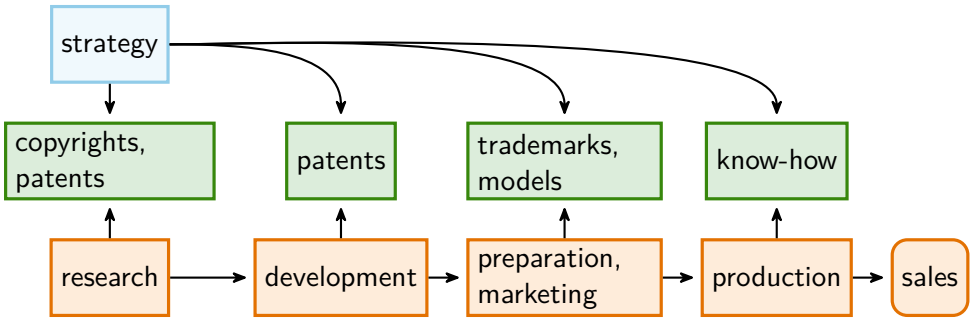

Figure 4.3: Generating IP with a new product

for (parts of) the manufacturing process. Such patented processes maybe out-licensed for example by companies in the chemical industry.

4.5 Purchasing and selling IP

In most economic sectors technologies are well developed at such a stage that many parts and processes are now available. Hence, there is no more reason to develop or manufacture those parts or processes. This is a huge difference compared with the upcoming economy at the start of the industrial revolution when manufacturers needed to have all parts and manufacturing processes in house by themselves. For example: the Ford Company wanted to have their own rubber plantations for the production of the tyres.

During the stage of research and development it is useful to analyse which technology, semi-finished products or parts can be purchased from others. Next the company can decide what needs further development by itself. Such strategy is also useful to identify interesting technologies developed by others which may solve technical problems and can be applied for further use. If these technologies have been appropriated in a patent portfolio of others they cannot be use as such without further analysis. Maybe there is a possibility to acquire ownership by assignment or come to terms in a license agreement.

4.5.1 Inlicensing patented technologies

A company may decide to obtain a license for a technology in order to start production and sales easier or faster. The results from a patent landscape analysis or Freedom to Operate may show that such a technology already exists or even that obtaining such a license agreement from a licensor is compulsory given the legal situation. Obviously, further information about the legal status on the validity of the patent in the country where the licensee

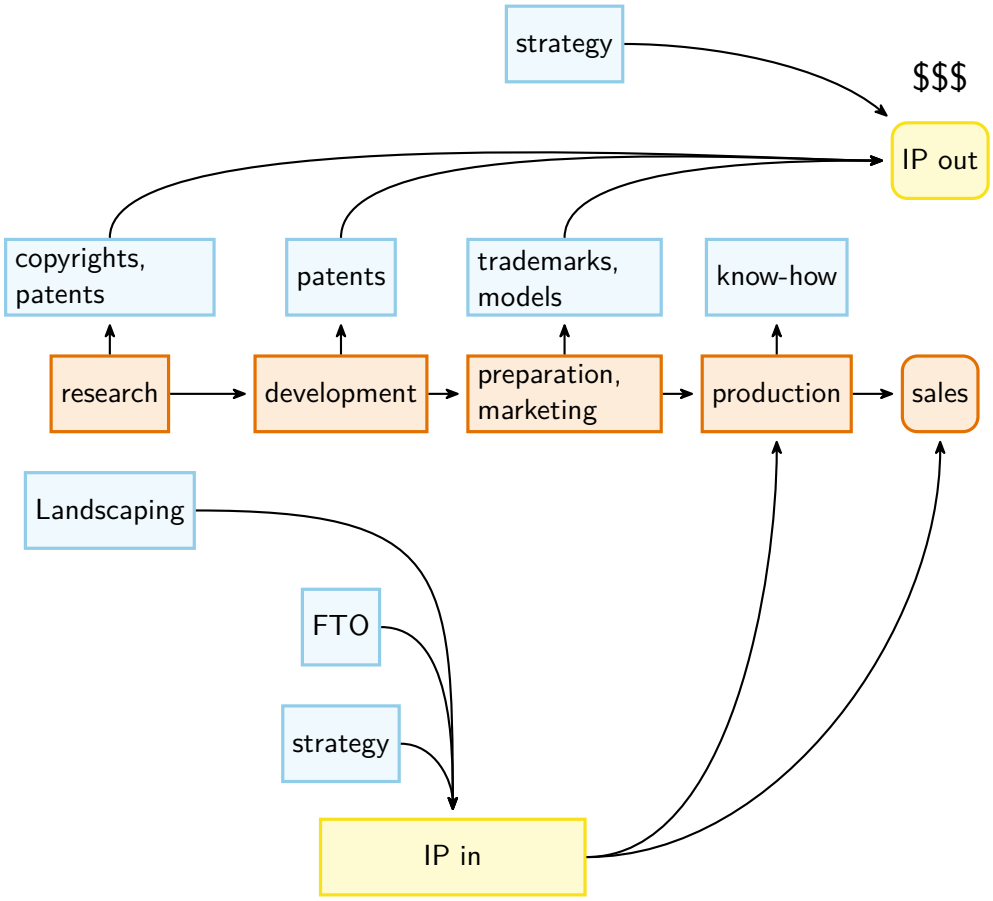

Figure 4.4: IE in and out

is operating is then required. For example if a Dutch manufacturer who is only working in the Netherlands needs certain technology the patent from the licensor should be valid in the Netherlands.

The business strategy and market perspectives are key in the decision making process to use licenses on technologies from third parties. But a patent landscape analysis is a useful business tool for companies with limited budgets for research and development. Next the company can contact the original patentee to start negotiations to obtain necessary patent licenses depending on its results. This is called inlicensing and presented as "IP in" in figure 4.4.

4.5.2 Outlicensing patented technologies

Usually a company decides to start production in a country or for a market by itself or by approaching others. Licensing technologies to others or franchising enables the patentee to do both. Such strategic decisions are often taken at central level of a multinational company or organisation and then followed up at decentral level.

But even if the patentee decides not to commercialise the technology itself, licensing to third parties remains an interesting option for example for organisations without production capacity in a particular country or market or a sales force. This is called outlicensing technologies and presented by "IP out" in figure 4.4. Outlicensing is often used successfully in cooperation with companies who are already active in certain markets and regions using the outlicensed technology to diversify their supply chain of products. Often the patentee is required to show successful sales records in an established home market for its patented technology.

4.5.3 Using patents in IP strategies

Depending on business strategy and use of IP a company can decide to outlicense their patent portfolio enabling others introducing new products or using manufacturing processes. Thereby allowing other companies to generate revenues without prior investments (in research and development, manufacturing, marketing, etc.) which were made by the patentee. This is called an offensive IP strategy which maybe more relevant for companies with products based upon a platform technology or compound with a large and diverse scope of applications.

On the other hand, companies may have a defensive IP strategy in the markets thereby stopping competitors selling look alike products to customers at lower prices. A large portfolio of nationally registered patented products in many countries is usually a prerequisite. Such a strategy may be relevant

for companies with patented products based upon very narrowly defined technologies and compounds which can easily be copied or circumvented.

Which IP strategy a company can use will depend on its market position at present and foreseeable future versus those of competitors. A patent landscape analysis gives interesting insights and a global overview on certain technical developments over the years. Such information is useful to determine the market position as defined by patents and can contribute in the decision making process which IP strategy best be followed. At the same time with this analysis one can retrieve information about the patent strategy of competitors.

4.6 Example of IP use of the PER.C6 technology

Crucell has used various IP rights to commercialize their PERC6 technology. In addition to their patents several IP rights like trademarks also played an important role. Following paragraphs describe the strategic use of their IP rights.

4.6.1 Patents

4.6.2 Purchase and assignment of patents

Chapter 5

Using IP for specific topics

5.1 Introduction

In this chapter the use of IP for specific topics is described. These topics are not linked to a specific activity as e.g. mentioned in chapter 4 and are of more general use.

Since software is nowadays very important in many parts of innovation and in society this topic is especially dealt with in section 5.2 and for open-source software in section 5.3.

5.2 Software

Computer programs are primarily protected by copyright.

Sometimes a patent can also be obtained on software-related inventions. Computer programs as such cannot be protected by patent law.

5.2.1 Copyright on software

Historically, there has been a long debate about whether software should be protected under patent law, copyright law or a separate legal regime. Ultimately, it was decided to protect software primarily under copyright law. This was a practical choice. Because software is written in programming language, it can be expressed as a kind of text. That is why computer programs are protected as literary works under copyright law. This principle is laid down in Article 10, paragraph 1, of the TRIPs Agreement and Article 4 of the WIPO Copyright Treaty.

Copyright protection of software relates to the concrete expression of the computer program, i.e. the specific form in which the programmer has expressed his intellectual creation in the source code. The source code concerns the instructions written by the programmer in a programming language and readable by humans. The target code is also subject to copyright protection. The object code comprises the binary, computer-readable and executable instructions generated from the source code by a compiler or interpreter. The object code is therefore in fact the translation of the source code into a computer-readable form.

The same conditions apply to copyright protection of software as to any other work. The source code and object code must demonstrate originality. They may not be derived from earlier software and the programmer must have made creative choices when writing the source code. If these conditions are met, the computer program is legally protected under copyright.[1]

Copyright does not protect an idea underlying a work. This means that the functionality, logic, method or purpose of a computer program and the processes, procedures, algorithms, programming languages and layout of data files that are used in the context of a computer program to be able to use certain functions of the program are not protected by copyright.

Copyright does not create a monopoly on the functionality of software. It grants the creator or right holder exclusive rights to permit or prohibit the reproduction (copying or editing) and publication (publishing, marketing, lending, renting or making available on demand) of a computer program. However, the creator or right holder cannot prohibit others from developing their own computer programs that pursue the same or similar purpose or functionality.

Copyright on software largely follows the same rules as those that apply to any other work. For example, the rules for authorship and legal succession are the same, right holders are entitled to the same broad exploitation rights and the term of protection is determined in the same way. However, there are a few special provisions concerning computer programs that are recorded in Chapter VI of the Copyright Act.

Based on the right of reproduction, the right holder may prohibit others from copying or taking over the computer program in whole or in part or from changing the source code. The law also stipulates that the right of reproduction also includes reproductions that are necessary for loading, displaying, executing, transmitting or storing the computer program. Someone who has lawfully obtained the software, such as the person who has purchased a computer program, may make these reproductions to the extent necessary

[1] Preparatory design material can also be protected by copyright, provided that no programming step with creative steps is needed to turn that material into a computer program.

for the use of the computer program. The lawful acquirer may also make a backup copy if this is necessary for the intended use.

In addition, the law permits the operation of software to be observed, studied and tested in order to discover the underlying ideas and principles. There is therefore an explicit authority to 'reverse-engineer' the software.

Furthermore, the 'decompilation' of a computer program, the reconstruction of a source code based on the target code, is permitted under certain circumstances. The law stipulates that a computer program may be decompiled, not in order to create a competing program that imitates the decompiled software, but to create compatible programs that can communicate with the decompiled software and are therefore interoperable. Furthermore, it follows from case law that decompilation is permitted to correct errors in the proper functioning of a computer program.

Graphical user interface and other elements

When executing a computer program on a computer, users are primarily confronted with the graphical user interface (GUI). These are the visual elements that enable the user to communicate with a computer program and thus instruct the program (software) to control the computer (hardware). Think of the various icons in the taskbar or the menu of a computer program.

However, the GUI itself is not a computer program. The special provisions regarding computer programs therefore do not apply to GUIs. A GUI can be independently protected by copyright, if the designer has made creative choices in the design of the interface. When decompiling a computer program for the purpose of interoperability or error correction, the source code may be reconstructed on the basis of the target code, but the GUI may not also be copied to the extent that it is protected by copyright. That would infringe the copyright on the GUI.

The same applies to the graphic and sound elements of, for example, video games. These can be independently protected by copyright if they are the creator's own intellectual creation, but do not themselves qualify as a computer program.

Video games

Video games generally consist of different types of works. In addition to software (source and target code), many video games contain a storyline, characters, images, animations, video, music and texts. Provided that the requirements are met, each of these

works enjoys copyright protection. In principle, the copyright on the various works can lie with different creators. Sometimes hundreds of people can have made a creative contribution to a single video game. Because permission must be obtained from each rights holder for the release of the video game, the large number of rights holders can greatly hinder exploitation.

In practice, it is therefore arranged that all copyrights on the video game are, as much as possible, in the hands of the producer of the video game. The Copyright Act already provides for this to some extent. Insofar as creators have contributed to a video game under employment, the copyrights are in principle already held by the producer as employer under the law. For components of a video game that have been created by freelancers on assignment, the producer will usually have the copyrights contractually transferred to him. In addition, the producer can stipulate that the creator waives the right to mention his name, so that the rights are automatically granted to his/her company. For existing works that are included in a video game, such as the music that plays in the background of a video game, the producer will usually arrange permission by concluding a license agreement with the relevant copyright holders.

5.2.2 Software patent law

The starting point of patent law is that software as such cannot be patented, because computer programs are not considered inventions. However, the term invention contains the requirement of technical nature. A computer program that has a 'further technical effect' when executed on a computer, beyond the effect of the normal control of the computer, can therefore be patented. The computer program must provide a technical solution to a technical problem. Inventions with software must also meet the patent law requirements of novelty, inventive step and industrial applicability (see section 3.5).

Examples of computer programs that have a 'further technical effect' when executed on a computer are programs for controlling an anti-lock braking system (ABS) in cars, determining emissions from X-ray equipment, compressing data, encrypting electronic communications, restoring distorted digital images or training artificial intelligence. A 'further technical effect' can also concern the internal functioning or security of the computer. For example, programs for distributing the processor load, memory allocation or securing integrity during start-up offer a technical solution to a technical problem.

Patent protection is broader than copyright protection in the sense that patent law does grant a temporary monopoly on the technical functionality of the software-related invention. Patent law gives the holder the exclusive right to prohibit others from applying and using the patented invention for commercial purposes. It is therefore not permitted to market computer programs with the same 'further technical effect', or an effect that is more or less equivalent, during the period that the patent is valid.

5.2.3 Other ways to protect software

In addition to copyright protection of computer programs and patent protection of software-related inventions, software or parts thereof can also be protected by other intellectual property rights. For example, the source code of computer programs can be protected as a trade secret. Graphic features of computer programs, such as icons or pictograms of the graphic user interface, can be protected as drawings under design and model law, provided of course that the specific protection conditions are met.

In addition, the producer of software can of course contractually agree on additional protection with third parties, for example in license agreements.

Software can of course also be protected technologically, by security measures such as encryption methods and copy protection. The Copyright Act offers protection against circumvention of such technological protection measures.

5.3 Example of IP use in open source software

Open source software, or alternatively also called free software (free as freedom and not necessarily free as in a free beer), aims to make the software available to everyone and to be developed jointly.

Part of this software is in the public domain and another part is licensed. Well-known licenses are the GPL (GNU General Public License) or the BSD (Berkely Software Distribution) license. These licenses allow the use of the software under certain conditions. The user must therefore comply with those conditions and is not free to do everything.

Question:

> How can the terms of the open source licenses be enforced if the source code is publicly available? Click for answer.

Although the open source movement mainly originated in the academic world, there are now many large companies that develop open source software. These companies use the joint development to offer products and services around the open source software.

5.4 Example of IP use with standards

5.4.1 VESA (Video Electronics Standards Association)

Vesa is a non-profit corporation, which represents more than 300 companies. These companies are members of the corporation. It sets and supports interface standards for computers and consumer electronics.

The vision statement (from the website):

> VESA's vision is continual growth in technical standards development and evolution into an international trade association, with world-wide membership driving standards initiatives, product implementations, and market implementation.

5.4.2 Displayport

The displayport connection between a computer and a monitor is an important Vesa standard. The Vesa members are allowed to use the displayport logo on their products if these meet the requirements of the standard. In figure 5.1 the logo is displayed.

Figure 5.1: Displayport logo

Question:

> How can the use of the logo be limited to members who comply to the standard? Click for answer.

Appendix A

Glossary

B

BOIP

Benelux Office for Intellectual Property. The Benelux Office for Intellectual Property (dutch BBIE: Benelux-Bureau voor de Intellectuele Eigendom, french: Office Benelux de la Propriété intellectuelle) registers trademarks and designs for the Benelux. 56

C

claims

The claims are part of a patent to define the scope of protection. Usually, the set of claims consists of a main claim with several dependent claims. 23, 26

D

diversify

Diversification gives companies the opportunity to expand their range of products and services. 43

DPMA

Deutsches Patent- und Markenamt. The German Patent and Trademark Office is tasked with the granting of patents and trademarks for Germany. 56

E

EPC

European Patent Convention. A multilateral treaty to provide the legal system for granting European patents. Next to articles and rules for obtaining a patent, it also institutes the European Patent Organisation. In German: EPÜ, French: CBE. The European Patent Office is tasked with the granting of the European patents. 19, 52, 62

EPO

European Patent Office. The European Patent Office is tasked with the granting of the European patents according the EPC. Main seat in Munich with dependancies in Rijswijk, Berlin and Vienna. 19, 26, 28, 56

EUIPO

European Union Intellectual Property Office. The European Union Intellectual Property Office registers trademarks and designs for the EU. 56

examiner

The person working at a patent office, who will do the substantive examination (search report and grant) of a patent application. 27

exhaustion

If a patent, trademark or design holder, or someone else with the permission of the holder, has sold a product, he can no longer use the patent, trademark or design right for that product. 20

F

Freedom to Operate

Freedom to Operate (FTO) is a study that analyzes potential risks of possible infringement of third party patents when introducing a new product to the market. 38, 39, 41

I

industrial property rights

Industrial property rights are all intellectual property rights except copyright. 36

innovation

Innovation is most often regarded as a new and tangible product or service which can be bought by customers in the market place. 36

intellectual properties

Intellectual property is a category of property that includes intangible creations of the human intellect. 6, 37, 38, 53

intellectual property rights

Intellectual Property Rights are the legal rights for creators over the creations of the minds. Intellectual property rights include patents, copyright, industrial design rights, trademarks, plant variety rights, trade dress, geographical indications, and in some jurisdictions trade secrets. 6, 53

IP

Intellectual Property. See also the description of intellectual properties and intellectual property rights in the glossary. 2, 6, 7, 9, 14, 37, 40, 43, 45, 53

IPR

Intellectual Property Rights are the legal rights for creators over the creations of their minds. See also the description of intellectual property rights and intellectual properties in the glossary. 6, 8, 9

J

JPO

Japan Patent Office. The Japan Patent Patent Office is tasked with the granting of patents and trademarks for Japan. 56

L

license

Meaning of license when used in IP: The right to commercially use a product or service to which another legal entity has intellectual property rights, on the basis of financial or material compensation. 41

O

Octrooicentrum Nederland

The Netherlands Patent Office is the patent office of the Netherlands. The Netherlands Patent Office is a department of the Netherlands Enterprise Agency, an agency of the Ministry of Economic Affairs and Climate Policy. The Netherlands Patent Office grants patents in the Netherlands and deals with European patents validated in the Netherlands. 19, 56

P

patent

A patent is an intellectual property right for an invention. 9, 18

patent landscape analysis

A patent landscape analysis provides a worldwide overview of patent holders who have technology in the economic sector of your organization. This gives you both market and product information of existing technology. With the help of this analysis, you can adjust research and development in time or decide to apply for a license from the patent holder for your market. 38, 39, 41, 44

PCT

Patent Cooperation Treaty. The Patent Cooperation Treaty is an international patent law treaty. It provides a unified procedure for filing patent applications to protect inventions in each of its contracting states. A patent application filed under the PCT is called an international application, or PCT application. 19, 24, 28, 30, 65

person skilled in the art

The term person skilled in the art, as used in patent law, is a constructed virtual person with knowledge and skill of a (broad) technical field. The person skilled in the art knows the entire state of the art, but has no inventive capacity. This constructed person skilled in the art is used in drawing up arguments, especially in the case of inventive step, sufficient disclosure and clarity of the patent application. 21–23

priority

A patent application can get right of priority from an earlier filing. This has the effect as if the patent application is filed on the date of the earlier filing. 31

R

ROW

National Patents Act 1995. Law for patents valid in the Netherlands, including the Caribbean, Curaçao and Sint Maarten. 19, 61

S

search report

The search report is prepared by the patent office where the patent application has been filed. It is used to assess novelty and inventive

step during the examination of the patent. It therefore contains the most relevant documents that are used in the examination. 27, 28

state of the art

The state of the art is formed by everything made available to the public by means of a written or oral description, by use, or in any other way, before the date of filing of the patent application 21, 27, 37

U

USPTO

United States Patent and Trademark Office. The United States Patent and Trademark Office is tasked with the granting of patents and trademarks for the United States of America. 56

W

WIPO

World Intellectual Property Organisation. The World Intellectual Property Organization is one of the 15 specialized agencies of the United Nations (UN). WIPO administers 26 international treaties that concern a wide variety of intellectual property issues, ranging from the protection of audiovisual works to establishing international patent classification. WIPO currently has 193 member states and is headquartered in Geneva, Switzerland. 19, 28, 56

Appendix B

Links

B.1 National and international IP offices

Netherlands patent office (Octrooicentrum Nederland):
https://www.rvo.nl/onderwerpen/innovatief-ondernemen/octrooien-ofwel-patenten

Benelux Office for Intellectual Property (BOIP):
https://www.boip.int/

European Patent Office (EPO):
https://www.epo.org/

European Union Intellectual Property Office (EUIPO):
https://www.euipo.europa.eu/

World Intellectual Property Organisation (WIPO):
https://www.wipo.int/

German patent office (DPMA):
https://www.dpma.de/

United States Patents and Trademark Office (USPTO):
https://www.uspto.gov/

Japan Patent Office (JPO):
https://www.jpo.go.jp/e/

B.2 Additional information

ThatsIP E-learning Intellectuel Property:
https://www.thatsip.nl/en/
Netherlands patent office, videos explaining basics of patents:
https://www.rvo.nl/onderwerpen/octrooien-ofwel-patenten/uitlegvideos

UK Intellectual Property Office, videos on IP basic, case studies and others:
https://www.youtube.com/user/ipogovuk

Werkgemeenschap Octrooi-informatie Nederland (WON):
http://www.won-nl.org

B.3 Interesting publications from the WIPO

What is Intellectual Property?
https://www.wipo.int/publications/en/details.jsp?id=4528&plang=EN

Intellectual Property Basics: A Q&A for Students
https://www.wipo.int/publications/en/details.jsp?id=4410&plang=EN

Understanding Industrial Property
https://www.wipo.int/publications/en/details.jsp?id=4080&plang=EN

Inventing the Future
An Introduction to Patents for Small and Medium-sized Enterprises
https://www.wipo.int/publications/en/details.jsp?id=4350&plang=EN

Enterprising Ideas
A Guide to Intellectual Property for Startups
https://www.wipo.int/publications/en/details.jsp?id=4545&plang=EN

Guide to the International Patent Classification (2022)
https://www.wipo.int/publications/en/details.jsp?id=4593&plang=EN

International Patent Classification (IPC)
https://www.wipo.int/publications/en/details.jsp?id=4582&plang=EN

B.4 IP databases

Espacenet:
https://worldwide.espacenet.com/patent/

Espacenet pocket guide:
https://www.epo.org/espacenet-pocket-guide

Manual Espacenet (Dutch):
https://www.rvo.nl/sites/default/files/2021/03/Handleiding%20Espacenet_februari2021.pdf

European Patent Register:
https://register.epo.org/

European Patent Bulletin:
https://data.epo.org/expert-services/index.html

Google patents:
https://patents.google.com/

Depatisnet (DPMA):
https://depatisnet.dpma.de/DepatisNet/depatisnet

Patentscope:
https://patentscope.wipo.int/

The lens:
https://www.lens.org/

Trademark view and Design view:
https://www.tmdn.org/

EUIPO register (eSearch plus):
https://euipo.europa.eu/eSearch/

BOIP trademark register:
https://www.boip.int/en/trademarks-register

BOIP design register:
https://www.boip.int/en/designs-register

Register of the Netherlands patent office:
https://mijnoctrooi.rvo.nl/fo-eregister-view/

Register of the German patent office (DPMA register):
https://register.dpma.de/DPMAregister/pat/basis

UK Intellectual Property Office, online patent information and document inspection service:
https://www.ipo.gov.uk/p-ipsum.htm

Japan platform for patent information:
https://www.j-platpat.inpit.go.jp/

B.5 The patent classification schemes

CPC classification scheme at the USPTO (US patent and trademark office):
https://www.uspto.gov/web/patents/classification/cpc/html/cpc.html

CPC classification scheme in table to download scheme and definitions:
https://www.cooperativepatentclassification.org/cpcSchemeAndDefinitions/table

Appendix C

Bibliography

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[GR10] C. Greenhalgh and M. Rogers. *Innovation, Intellectual Property, and Economic Growth*. Princeton University Press, 2010. ISBN: 9781400832231. URL: https://press.princeton.edu/books/paperback/9780691137995/innovation-intellectual-property-and-economic-growth.

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Appendix D

Parts of IP law

D.1 Parts of the Dutch patent law, Rijksoctrooiwet 1995 (in Dutch)

These are some of the most relevant parts of Dutch patent law (ROW).

- Artikel 53
    1. Een octrooi geeft de octrooihouder, behoudens de bepalingen van de artikelen 53a tot en met 60, het uitsluitend recht:
        a. het geoctrooieerde voortbrengsel in of voor zijn bedrijf te vervaardigen, te gebruiken, in het verkeer te brengen of verder te verkopen, te verhuren, af te leveren of anderszins te verhandelen, dan wel voor een of ander aan te bieden, in te voeren of in voorraad te hebben;
        b. de geoctrooieerde werkwijze in of voor zijn bedrijf toe te passen of het voortbrengsel, dat rechtstreeks verkregen is door toepassing van die werkwijze, in of voor zijn bedrijf te gebruiken, in het verkeer te brengen of verder te verkopen, te verhuren, af te leveren of anderszins te verhandelen, dan wel voor een of ander aan te bieden, in te voeren of in voorraad te hebben.
    2. Het uitsluitend recht wordt bepaald door de conclusies van het octrooischrift, waarbij de beschrijving en de tekeningen dienen tot uitleg van die conclusies.
    3. Het uitsluitend recht strekt zich niet uit over handelingen, uitsluitend dienende tot onderzoek van het geoctrooieerde, daaronder begrepen het door toepassing van de geoctrooieerde werkwijze rechtstreeks verkregen voortbrengsel. Het uitsluitend recht strekt zich evenmin uit tot de bereiding voor direct gebruik ten

behoeve van individuele gevallen op medisch voorschrift van geneesmiddelen in apotheken, noch tot handelingen betreffende de aldus bereide geneesmiddelen.

4. Het uitvoeren van de noodzakelijke studies, tests en proeven met het oog op de toepassing van artikel 10, eerste tot en met vierde lid, van Richtlijn 2001/83/EG tot vaststelling van een communautair wetboek betreffende geneesmiddelen voor menselijk gebruik (PbEG L 311) of artikel 13, eerste tot en met het vijfde lid van Richtlijn 2001/82/EG tot vaststelling van een communautair wetboek betreffende geneesmiddelen voor diergeneeskundig gebruik (PbEG L 311) en de daaruit voortvloeiende praktische vereisten worden niet beschouwd als een inbreuk op octrooien met betrekking tot geneesmiddelen voor menselijk gebruik, respectievelijk geneesmiddelen voor diergeneeskundig gebruik.
5. Is een voortbrengsel als in het eerste lid, onder a of b, bedoeld, in Nederland, Curaçao of Sint Maarten rechtmatig in het verkeer gebracht, dan wel door de octrooihouder of met diens toestemming in één der Lid-Staten van de Europese Gemeenschap of in een andere staat die partij is bij de Overeenkomst betreffende de Europese Economische Ruimte in het verkeer gebracht, dan handelt de verkrijger of latere houder niet in strijd met het octrooi, door dit voortbrengsel in of voor zijn bedrijf te gebruiken, te verkopen, te verhuren, af te leveren of anderszins te verhandelen, dan wel voor een of ander aan te bieden, in te voeren of in voorraad te hebben.
6. Een voortbrengsel als in het eerste lid, onder a of b, bedoeld, dat voor de verlening van het octrooi, of, indien het een Europees octrooi betreft, voor de dag, waarop overeenkomstig artikel 97, derde lid, van het Europees Octrooiverdrag de vermelding van de verlening van het Europees octrooi is gepubliceerd, in een bedrijf is vervaardigd, mag niettegenstaande het octrooi ten dienste van dat bedrijf worden gebruikt.

D.2 Parts of the European Patent Convention

These are some of the most relevant parts of patent law in the European Patent Convention (EPC).

- Article 52. Patentable inventions

  (1) European patents shall be granted for any inventions, in all fields of technology, provided that they are new, involve an inventive step and are susceptible of industrial application.

(2) The following in particular shall not be regarded as inventions within the meaning of paragraph 1:
   a) discoveries, scientific theories and mathematical methods;
   b) aesthetic creations;
   c) schemes, rules and methods for performing mental acts, playing games or doing business, and programs for computers;
   d) presentations of information.

(3) Paragraph 2 shall exclude the patentability of the subject-matter or activities referred to therein only to the extent to which a European patent application or European patent relates to such subject-matter or activities as such.

- Article 54. Novelty

(1) An invention shall be considered to be new if it does not form part of the state of the art.

(2) The state of the art shall be held to comprise everything made available to the public by means of a written or oral description, by use, or in any other way, before the date of filing of the European patent application.

(3) Additionally, the content of European patent applications as filed, the dates of filing of which are prior to the date referred to in paragraph 2 and which were published on or after that date, shall be considered as comprised in the state of the art.

(4) Paragraphs 2 and 3 shall not exclude the patentability of any substance or composition, comprised in the state of the art, for use in a method referred to in Article 53(c), provided that its use for any such method is not comprised in the state of the art.

(5) Paragraphs 2 and 3 shall also not exclude the patentability of any substance or composition referred to in paragraph 4 for any specific use in a method referred to in Article 53(c), provided that such use is not comprised in the state of the art.

- Article 56. Inventive step

An invention shall be considered as involving an inventive step if, having regard to the state of the art, it is not obvious to a person skilled in the art. If the state of the art also includes documents within the meaning of Article 54, paragraph 3, these documents shall not be considered in deciding whether there has been an inventive step.

- Article 83. Disclosure of the invention

The European patent application shall disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

- Article 84. Claims

  The claims shall define the matter for which protection is sought. They shall be clear and concise and be supported by the description.

- Article 87. Priority right

  (1) Any person who has duly filed, in or for
  (a) any State party to the Paris Convention for the Protection of Industrial Property or
  (b) any Member of the World Trade Organization,
  an application for a patent, a utility model or a utility certificate, or his successor in title, shall enjoy, for the purpose of filing a European patent application in respect of the same invention, a right of priority during a period of twelve months from the date of filing of the first application.
  (2) Every filing that is equivalent to a regular national filing under the national law of the State where it was made or under bilateral or multilateral agreements, including this Convention, shall be recognised as giving rise to a right of priority.
  (3) A regular national filing shall mean any filing that is sufficient to establish the date on which the application was filed, whatever the outcome of the application may be.
  (4) A subsequent application in respect of the same subject-matter as a previous first application and filed in or for the same State shall be considered as the first application for the purposes of determining priority, provided that, at the date of filing the subsequent application, the previous application has been withdrawn, abandoned or refused, without being open to public inspection and without leaving any rights outstanding, and has not served as a basis for claiming a right of priority. The previous application may not thereafter serve as a basis for claiming a right of priority.
  (5) If the first filing has been made with an industrial property authority which is not subject to the Paris Convention for the Protection of Industrial Property or the Agreement Establishing the World Trade Organization, paragraphs 1 to 4 shall apply if that authority, according to a communication issued by the President of the European Patent Office, recognises that a first filing made with the European Patent Office gives rise to a right of priority under conditions and with effects equivalent to those laid down in the Paris Convention.

- Article 88. Claiming priority

  (1) An applicant desiring to take advantage of the priority of a previous application shall file a declaration of priority and any other

document required, in accordance with the Implementing Regulations.

(2) Multiple priorities may be claimed in respect of a European patent application, notwithstanding the fact that they originated in different countries. Where appropriate, multiple priorities may be claimed for any one claim. Where multiple priorities are claimed, time limits which run from the date of priority shall run from the earliest date of priority.

(3) If one or more priorities are claimed in respect of a European patent application, the right of priority shall cover only those elements of the European patent application which are included in the application or applications whose priority is claimed.

(4) If certain elements of the invention for which priority is claimed do not appear among the claims formulated in the previous application, priority may nonetheless be granted, provided that the documents of the previous application as a whole specifically disclose such elements.

- Article 89. Effect of priority right

  The right of priority shall have the effect that the date of priority shall count as the date of filing of the European patent application for the purposes of Article 54, paragraphs 2 and 3, and Article 60, paragraph 2.

D.3 Parts of the Patent Cooperation Treaty

These are some of the most relevant parts of Patent Cooperation Treaty (PCT).

- Article 5. The Description

  The description shall disclose the invention in a manner sufficiently clear and complete for the invention to be carried out by a person skilled in the art.

- Article 6. The Claims

  The claim or claims shall define the matter for which protection is sought. Claims shall be clear and concise. They shall be fully supported by the description.

- Article 8. Claiming Priority

  (1) The international application may contain a declaration, as prescribed in the Regulations, claiming the priority of one or more

earlier applications filed in or for any country party to the Paris Convention for the Protection of Industrial Property.

(2) (a) Subject to the provisions of subparagraph (b), the conditions for, and the effect of, any priority claim declared under paragraph (1) shall be as provided in Article 4 of the Stockholm Act of the Paris Convention for the Protection of Industrial Property

(b) The international application for which the priority of one or more earlier applications filed in or for a Contracting State is claimed may contain the designation of that State. Where, in the international application, the priority of one or more national applications filed in or for a designated State is claimed, or where the priority of an international application having designated only one State is claimed, the conditions for, and the effect of, the priority claim in that State shall be governed by the national law of that State.

- Article 33. The International Preliminary Examination

(1) The objective of the international preliminary examination is to formulate a preliminary and non-binding opinion on the questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), and to be industrially applicable.

(2) For the purposes of the international preliminary examination, a claimed invention shall be considered novel if it is not anticipated by the prior art as defined in the Regulations.

(3) For the purposes of the international preliminary examination, a claimed invention shall be considered to involve an inventive step if, having regard to the prior art as defined in the Regulations, it is not, at the prescribed relevant date, obvious to a person skilled in the art.

(4) For the purposes of the international preliminary examination, a claimed invention shall be considered industrially applicable if, according to its nature, it can be made or used (in the technological sense) in any kind of industry. "Industry" shall be understood in its broadest sense, as in the Paris Convention for the Protection of Industrial Property.

(5) The criteria described above merely serve the purposes of international preliminary examination. Any Contracting State may apply additional or different criteria for the purpose of deciding whether, in that State, the claimed invention is patentable or not.

(6) The international preliminary examination shall take into consideration all the documents cited in the international search report.

It may take into consideration any additional documents considered to be relevant in the particular case.

- Rule 64. Prior Art for International Preliminary Examination

  64.1 Prior Art

  (a) For the purposes of Article 33(2) and (3), everything made available to the public anywhere in the world by means of written disclosure (including drawings and other illustrations) shall be considered prior art provided that such making available occurred prior to the relevant date.
  (b) For the purposes of paragraph (a), the relevant date shall be:
    (i) subject to item (ii) and (iii), the international filing date of the international application under international preliminary examination;
    (ii) where the international application under international preliminary examination claims the priority of an earlier application and has an international filing date which is within the priority period, the filing date of such earlier application, unless the International Preliminary Examining Authority considers that the priority claim is not valid;
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In cases where any application or any patent which would constitute prior art for the purposes of Article 33(2) and (3) had it been published prior to the relevant date referred to in Rule 64.1 was published on a date which is the same as, or later than, the relevant date but was filed earlier than the relevant date or claimed the priority of an earlier application which had been filed prior to the relevant date, such published application or patent shall not be considered part of the prior art for the purposes of Article 33(2) and (3). Nevertheless, the international preliminary examination report shall call attention to such application or patent in the manner provided for in Rule 70.10.

Appendix E

Documents

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(54) Title: PACKAGING SYSTEMS FOR HUMAN RECOMBINANT ADENOVIRUS TO BE USED IN GENE THERAPY

(57) Abstract

The invention provides improved methods and products based on adenoviral materials which can advantageously be used in for instance gene therapy. In one aspect an adenoviral vector is provided which has no overlap with a suitable packaging cell line which is another aspect of invention. This combination excludes the possibility of homologous recombination, thereby excluding the possibility of the formation of replication competent adenovirus. In another aspect an adenovirus based helper construct which by its size is incapable of being encapsidated. This helper virus can be transferred into any suitable host cell making it a packaging cell. Further a number of useful mutations to adenoviral based materials and combinations of such mutations are disclosed, which all have in common the safety of the methods and the products, in particular avoiding the production of replication competent adenovirus and/or interference with the immune system. Further a method of intracellular amplification is provided.

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Title: **Packaging systems for human recombinant adenovirus to be used in gene therapy.**

The invention relates to the field of recombinant DNA technology, more in particular to the field of gene therapy. In particular the invention relates to gene therapy using materials derived from adenovirus, in particular human recombinant adenovirus. It especially relates to novel virus derived vectors and novel packaging cell lines for vectors based on adenoviruses.

Gene therapy is a recently developed concept for which a wide range of applications can be and have been envisaged.

In gene therapy a molecule carrying genetic information is introduced into some or all cells of a host, as a result of which the genetic information is added to the host in a functional format.

The genetic information added may be a gene or a derivative of a gene, such as a cDNA, which encodes a protein. In this case the functional format means that the protein can be expressed by the machinery of the host cell.

The genetic information can also be a sequence of nucleotides complementary to a sequence of nucleotides (be it DNA or RNA) present in the host cell. The functional format in this case is that the added DNA (nucleic acid) molecule or copies made thereof in situ are capable of base pairing with the complementary sequence present in the host cell.

Applications include the treatment of genetic disorders by supplementing a protein or other substance which is, through said genetic disorder, not present or at least present in insufficient amounts in the host, the treatment of tumors and (other) acquired diseases such as (auto)immune diseases or infections, etc.

As may be clear from the above, there are basically three different approaches in gene therapy, one directed towards compensating a deficiency present in a (mammalian) host; the second directed towards the removal or elimination of unwanted substances (organisms or cells) and the third towards application of a recombinant vaccine (tumors or foreign micro-organisms).

For the purpose of gene therapy, adenoviruses carrying deletions have been proposed as suitable vehicles. Adenoviruses are non-enveloped DNA viruses. Gene-transfer vectors derived from adenoviruses (so-called adenoviral vectors) have a number of features that make them particularly useful for gene transfer for such purposes. Eg. the biology of the adenoviruses is characterized in detail, the adenovirus is not associated with severe human pathology, the virus is extremely efficient in introducing its DNA into the host cell, the virus can infect a wide variety of cells and has a broad host-range, the virus can be produced in large quantities with relative ease, and the virus can be rendered replication defective by deletions in the early-region 1 (E1) of the viral genome.

The adenovirus genome is a linear double-stranded DNA molecule of approximately 36000 base pairs with the 55-kDa terminal protein covalently bound to the 5'terminus of each strand. The Ad DNA contains identical Inverted Terminal Repeats (ITR) of about 100 base pairs with the exact length depending on the serotype. The viral origins of replication are located within the ITRs exactly at the genome ends. DNA synthesis occurs in two stages. First, the replication proceeds by strand displacement, generating a daughter duplex molecule and a parental displaced strand. The displaced strand is single stranded and can form a so-called "panhandle" intermediate, which allows replication initiation and generation of a daughter duplex molecule. Alternatively, replication may proceed from both ends of the genome simultaneously, obviating the requirement to form the

panhandle structure. The replication is summarized in Figure 14 adapted from (Lechner and Kelly, 1977).

During the productive infection cycle, the viral genes are expressed in two phases: the early phase, which is the period upto viral DNA replication, and the late phase, which coincides with the initiation of viral DNA replication. During the early phase only the early gene products, encoded by regions E1, E2, E3 and E4, are expressed, which carry out a number of functions that prepare the cell for synthesis of viral structural proteins (Berk, 1986). During the late phase the late viral gene products are expressed in addition to the early gene products and host cell DNA and protein synthesis are shut off. Consequently, the cell becomes dedicated to the production of viral DNA and of viral structural proteins (Tooze, 1981).

The E1 region of adenovirus is the first region of adenovirus expressed after infection of the target cell. This region consists of two transcriptional units, the E1A and E1B genes, which both are required for oncogenic transformation of primary (embryonal) rodent cultures. The main functions of the E1A gene products are:
i) to induce quiescent cells to enter the cell cycle and resume cellular DNA synthesis, and
ii) to transcriptionally activate the E1B gene and the other early regions (E2, E3, E4). Transfection of primary cells with the E1A gene alone can induce unlimited proliferation (immortalization), but does not result in complete transformation. However, expression of E1A in most cases results in induction of programmed cell death (apoptosis), and only occasionally immortalization is obtained (Jochemsen et al., 1987). Co-expression of the E1B gene is required to prevent induction of apoptosis and for complete morphological transformation to occur. In established immortal cell lines, high level expression of E1A can cause complete transformation in the absence of E1B (Roberts et al., 1985).

The E1B encoded proteins assist E1A in redirecting the cellular functions to allow viral replication. The E1B 55 kD and E4 33kD proteins, which form a complex that is essentially localized in the nucleus, function in inhibiting the synthesis of host proteins and in facilitating the expression of viral genes. Their main influence is to establish selective transport of viral mRNAs from the nucleus to the cytoplasm, concomittantly with the onset of the late phase of infection. The E1B 21 kD protein is important for correct temporal control of the productive infection cycle, thereby preventing premature death of the host cell before the virus life cycle has been completed. Mutant viruses incapable of expressing the E1B 21 kD gene-product exhibit a shortened infection cycle that is accompanied by excessive degradation of host cell chromosomal DNA (*deg*-phenotype) and in an enhanced cytopathic effect (*cyt*-phenotype) (Telling et al., 1994). The *deg* and *cyt* phenotypes are suppressed when in addition the E1A gene is mutated, indicating that these phenotypes are a function of E1A (White et al., 1988). Furthermore, the E1B 21 kDa protein slows down the rate by which E1A switches on the other viral genes. It is not yet known through which mechanisms) E1B 21 kD quenches these E1A dependent functions.

Vectors derived from human adenoviruses, in which at least the E1 region has been deleted and replaced by a gene of interest, have been used extensively for gene therapy experiments in the pre-clinical and clinical phase.

As stated before all adenovirus vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Stratford-Perricaudet and Perricaudet, 1991). We have demonstrated that recombinant adenoviruses are able to efficiently transfer recombinant genes to the rat liver and airway epithelium of rhesus monkeys (Bout et

al., 1994b; Bout et al., 1994a). In addition, we (Vincent et al., 1996a; Vincent et al., 1996b) and others (see e.g. Haddada et al., 1993) have observed a very efficient *in vivo* adenovirus mediated gene transfer to a variety of tumor cells *in vitro* and to solid tumors in animals models (lung tumors, glioma) and human xenografts in immunodeficient mice (lung) *in vivo* (reviewed by Blaese et al., 1995).

In contrast to for instance retroviruses, adenoviruses a) do not integrate into the host cell genome; b) are able to infect non-dividing cells and c) are able to efficiently transfer recombinant genes *in vivo* (Brody and Crystal, 1994). Those features make adenoviruses attractive candidates for *in vivo* gene transfer of, for instance, suicide or cytokine genes into tumor cells.

However, a problem associated with current recombinant adenovirus technology is the possibility of unwanted generation of replication competent adenovirus (RCA) during the production of recombinant adenovirus (Lochmüller et al., 1994; Imler et al., 1996). This is caused by homologous recombination between overlapping sequences from the recombinant vector and the adenovirus constructs present in the complementing cell line, such as the 293 cells (Graham et al., 1977). RCA in batches to be used in clinical trials is unwanted because RCA i) will replicate in an uncontrolled fashion; ii) can complement replication defective recombinant adenovirus, causing uncontrolled multiplication of the recombinant adenovirus and iii) batches containing RCA induce significant tissue damage and hence strong pathological side effects (Lochmüller et al., 1994). Therefore, batches to be used in clinical trials should be proven free of RCA (Ostrove, 1994). In one aspect of the invention this problem in virus production is solved in that we have developed packaging cells that have no overlapping sequences with a new basic vector and thus are suited for safe large scale

production of recombinant adenoviruses one of the additional problems associated with the use of recombinant adenovirus vectors is the host-defence reaction against treatment with adenovirus.

Briefly, recombinant adenoviruses are deleted for the E1 region (see above). The adenovirus E1 products trigger the transcription of the other early genes (E2, E3, E4), which consequently activate expression of the late virus genes. Therefore, it was generally thought that E1 deleted vectors would not express any other adenovirus genes. However, recently it has been demonstrated that some cell types are able to express adenovirus genes in the absence of E1 sequences. This indicates, that some cell types possess the machinery to drive transcription of adenovirus genes. In particular, it was demonstrated that such cells synthesize E2A and late adenovirus proteins.

In a gene therapy setting, this means that transfer of the therapeutic recombinant gene to somatic cells not only results in expression of the therapeutic protein but may also result in the synthesis of viral proteins. Cells that express adenoviral proteins are recognized and killed by Cytotoxic T Lymphocytes, which thus a) eradicates the transduced cells and b) causes inflammations (Bout et al., 1994a; Engelhardt et al., 1993; Simon et al., 1993).As this adverse reaction is hampering gene therapy, several solutions to this problem have been suggested, such as a) using immunosuppressive agents after treatment; b) retainment of the adenovirus E3 region in the recombinant vector (see patent application EP 95202213) and c) and using ts mutants of human adenovirus, which have a point mutation in the E2A region (patent WO/28938).

However, these strategies to circumvent the immune response have their limitations.

The use of ts mutant recombinant adenovirus diminishes the immune response to some extent, but was less effective in preventing pathological responses in the lungs (Engelhardt et al., 1994a).

The E2A protein may induce an immune response by itself and it plays a pivotal role in the switch to the synthesis of late adenovirus proteins. Therefore, it is attractive to make recombinant adenoviruses which are mutated in the E2 region, rendering it temperature sensitive (ts), as has been claimed in patent application WO/28938.

A major drawback of this system is the fact that, although the E2 protein is unstable at the non-permissive temperature, the immunogenic protein is still being synthesized. In addition, it is to be expected that the unstable protein does activate late gene expression, albeit to a low extent. ts125 mutant recombinant adenoviruses have been tested, and prolonged recombinant gene expression was reported (Yang et al., 1994b; Engelhardt et al., 1994a; Engelhardt et al., 1994b; Yang et al., 1995). However, pathology in the lungs of cotton rats was still high (Engelhardt et al., 1994a), indicating that the use of ts mutants results in only a partial improvement in recombinant adenovirus technology. Others (Fang et al., 1996) did not observe prolonged gene expression in mice and dogs using ts125 recombinant adenovirus. An additional difficulty associated with the use of ts125 mutant adenoviruses is that a high frequency of reversion is observed. These revertants are either real revertants or the result of second site mutations (Kruijer et al., 1983; Nicolas et al., 1981). Both types of revertants have an E2A protein that functions at normal temperature and have therefore similar toxicity as the wild-type virus.

In another aspect of the present invention we therefore delete E2A coding sequences from the recombinant adenovirus genome and transfect these E2A sequences into the (packaging) cell lines containing E1 sequences to complement recombinant adenovirus vectors.

Major hurdles in this approach are a) that E2A should be expressed to very high levels and b) that E2A protein is very toxic to cells.

The current invention in yet another aspect therefore discloses use of the ts125 mutant E2A gene, which produces a protein that is not able to bind DNA sequences at the non permissive temperature. High levels of this protein may be maintained in the cells (because it is not toxic at this temperature) until the switch to the permissive temperature is made. This can be combined with placing the mutant E2A gene under the direction of an inducible promoter, such as for instance tet, methallothionein, steroid inducible promoter, retinoic acid β-receptor or other inducible systems. However in yet another aspect of the invention, the use of an inducible promoter to control the moment of production of toxic wild-type E2A is disclosed.

Two salient additional advantages of E2A-deleted recombinant adenovirus are the increased capacity to harbor heterologous sequences and the permanent selection for cells that express the mutant E2A. This second advantage relates to the high frequency of reversion of ts125 mutation: when reversion occurs in a cell line harboring ts125 E2A, this will be lethal to the cell. Therefore, there is a permanent selection for those cells that express the ts125 mutant E2A protein. In addition, as we in one aspect of the invention generate E2A-deleted recombinant adenovirus, we will not have the problem of reversion in our adenoviruses.

In yet another aspect of the invention as a further improvement the use of non-human cell lines as packaging cell lines is disclosed.

For GMP production of clinical batches of recombinant viruses it is desirable to use a cell line that has been used widely for production of other biotechnology products. Most of the latter cell lines are from monkey origin, which have been used to produce e.g. vaccines.

These cells can not be used directly for the production of recombinant human adenovirus, as human adenovirus can not or only to low levels replicate in cells of monkey origin. A block in the switch of early to late phase of adenovirus lytic cycle is underlying defective replication. However, host range mutations in the human adenovirus genome are described (hr400 - 404) which allow replication of human viruses in monkey cells. These mutations reside in the gene encoding E2A protein (Klessig and Grodzicker, 1979; Klessig et al., 1984; Rice and Klessig, 1985)(Klessig et al., 1984). Moreover, mutant viruses have been described that harbor both the hr and temperature-sensitive ts125 phenotype (Brough et al., 1985; Rice and Klessig, 1985).

We therefore generate packaging cell lines of monkey origin (e.g. VERO, CV1) that harbor:

a. E1 sequences, to allow replication of E1/E2 defective adenoviruses, and
b. E2A sequences, containing the hr mutation and the ts 125 mutation, named ts400 (Brough et al., 1985; Rice and Klessig, 1985) to prevent cell death by E2A overexpression, and/or
c. E2A sequences, just containing the hr mutation, under the control of an inducible promoter, and/or
d. E2A sequences, containing the hr mutation and the ts 125 mutation (ts400), under the control of an inducible promoter

Furthermore we disclose the construction of novel and improved combinations of (novel and improved) packaging cell lines and (novel and improved) recombinant adenovirus vectors. We provide:

1. a novel packaging cell line derived from diploid human embryonic retinoblasts (HER) that harbors nt. 80 - 5788 of the Ad5 genome.This cell line, named 911, deposited under no 95062101 at the ECACC, has many characteristics that make it superior to the commonly used 293 cells (Fallaux et al., 1996).

2. novel packaging cell lines that express just E1A genes and not E1B genes.
Established cell lines (and not human diploid cells of which 293 and 911 cells are derived) are able to express E1A to high levels without undergoing apoptotic cell death, as occurs in human diploid cells that express E1A in the absence of E1B.
Such cell lines are able to trans-complement E1B-defective recombinant adenoviruses, because viruses mutated for E1B 21 kD protein are able to complete viral replication even faster than wild-type adenoviruses (Telling et al., 1994). The constructs are described in detail below, and graphically represented in Figures 1-5. The constructs are transfected into the different established cell lines and are selected for high expression of E1A. This is done by operatively linking a selectable marker gene (e.g. NEO gene) directly to the E1B promoter. The E1B promoter is transcriptionally activated by the E1A gene product and therefore resistance to the selective agent (e.g. G418 in the case NEO is used as the selection marker) results in direct selection for desired expression of the E1A gene

3 Packaging constructs that are mutated or deleted for E1B 21 kD, but just express the 55 kD protein.

4. Packaging constructs to be used for generation of complementing packaging cell lines from diploid cells (not exclusively of human origin) without the need of selection with marker genes. These cells are immortalized by expression of E1A. However, in this particular case expression of E1B is essential to prevent apoptosis induced by E1A proteins.
Selection of E1 expressing cells is achieved by selection for focus formation (immortalization), as described for 293 cells (Graham et al., 1977) and 911 cells (Fallaux et al, 1996), that are E1-transformed

human embryonic kidney (HEK) cells and human embryonic retinoblasts (HER), respectively.

5. After transfection of HER cells with construct pIG.E1B (Fig. 4), seven independent cell lines could be established. These cell lines were designated PER.C1, PER.C3, PER.C4, PER.C5, PER.C6, PER.C8 and PER.C9. PER denotes PGK-E1-Retinoblasts. These cell lines express E1A and E1B proteins, are stable (e.g. PER.C6 for more than 57 passages) and complement E1 defective adenovirus vectors. Yields of recombinant adenovirus obtained on PER cells are a little higher than obtained on 293 cells. One of these cell lines (PER.C6) has been deposited at the ECACC under number 96022940.

6. New adenovirus vectors with extended E1 deletions (deletion nt. 459 - 3510). Those viral vectors lack sequences homologous to E1 sequences in said packaging cell lines. These adenoviral vectors contain pIX promoter sequences and the pIX gene, as pIX (from its natural promoter sequences) can only b expressed from the vector and not by packaging cells (Matsui et al , 1986, Hoeben and Fallaux, pers.comm.; Imler et al., 1996).

7. E2A expressing packaging cell lines preferably based on either E1A expressing established cell lines or E1A - E1B expressing diploid cells (see under 2 - 4). E2A expression is either under the control of an inducible promoter or the E2A ts125 mutant is driven by either an inducible or a constitutive promoter.

8. Recombinant adenovirus vectors as described before (see 6) but carrying an additional deletion of E2A sequences.

9. Adenovirus packaging cells from monkey origin that are able to trans-complement E1-defective recombinant adenoviruses. They are preferably co-transfected with pIG.E1AE1B and pIG.NEO, and selected for NEO resistance. Such cells expressing E1A and E1B are able

to transcomplement E1 defective recombinant human adenoviruses, but will do so inefficiently because of a block of the synthesis of late adenovirus proteins in cells of monkey origin (Klessig and Grodzicker, 1979). To overcome this problem, we generate recombinant adenoviruses that harbor a host-range mutation in the E2A gene, allowing human adenoviruses to replicate in monkey cells. Such viruses are generated as described in Figure 12, except DNA from a hr-mutant is used for homologous recombination.

10. Adenovirus packaging cells from monkey origin as described under 9, except that they will also be co-transfected with E2A sequences harboring the hr mutation. This allows replication of human adenoviruses lacking E1 and E2A (see under 8). E2A in these cell lines is either under the control of an inducible promoter or the tsE2A mutant is used. In the latter case, the E2A gene will thus carry both the ts mutation and the hr mutation (derived from ts400). Replication competent human adenoviruses have been described that harbor both mutations (Brough et al., 1985; Rice and Klessig, 1985).

A further aspect of the invention provides otherwise improved adenovirus vectors, as well as novel strategies for generation and application of such vectors and a method for the intracellular amplification of linear DNA fragments in mammalian cells.

The so-called "minimal" adenovirus vectors according to the present invention retain at least a portion of the viral genome that is required for encapsidation of the genome into virus particles (the encapsidation signal), as well as at least one copy of at least a functional part or a derivative of the Inverted Terminal Repeat (ITR), that is DNA sequences derived from the termini of the linear adenovirus genome. The vectors according to the present invention will also contain a transgene linked to a

promoter sequence to govern expression of the transgene. Packaging of the so-called minimal adenovirus vector can be achieved by co-infection with a helper virus or, alternatively, with a packaging deficient replicating helper system as described below.

Adenovirus-derived DNA fragments that can replicate in suitable cell lines and that may serve as a packaging deficient replicating helper system are generated as follows. These DNA fragments retain at least a portion of the transcribed region of the "late" transcription unit of the adenovirus genome and carry deletions in at least a portion of the E1 region and deletions in at least a portion of the encapsidation signal. In addition, these DNA fragments contain at least one copy of an inverted terminal repeat (ITR). At one terminus of the transfected DNA molecule an ITR is located. The other end may contain an ITR, or alternatively, a DNA sequence that is complementary to a portion of the same strand of the DNA molecule other than the ITR. If, in the latter case, the two complementary sequences anneal, the free 3'-hydroxyl group of the 3' terminal nucleotide of the hairpin-structure can serve as a primer for DNA synthesis by cellular and/or adenovirus-encoded DNA polymerases, resulting in conversion into a double-stranded form of at least a portion of the DNA molecule. Further replication initiating at the ITR will result in a linear double-stranded DNA molecule, that is flanked by two ITR's, and is larger than the original transfected DNA molecule (see Fig. 13). This molecule can replicate itself in the transfected cell by virtue of the adenovirus proteins encoded by the DNA molecule and the adenoviral and cellular proteins encoded by genes in the host-cell genome. This DNA molecule can not be encapsidated due to its large size (greater than 39000 base pairs) or due to the absence of a functional encapsidation signal. This DNA molecule is intended to serve as a helper for the

production of defective adenovirus vectors in suitable cell lines.

The invention also comprises a method for the amplification of linear DNA fragments of variable size in suitable mammalian cells. These DNA fragments contain at least one copy of the ITR at one of the termini of the fragment. The other end may contain an ITR, or alternatively, a DNA sequence that is complementary to a portion of the same strand of the DNA molecule other than the ITR. If, in the latter case, the two complementary sequences anneal, the free 3'-hydroxyl group of the 3' terminal nucleotide of the hairpin-structure can serve as a primer for DNA synthesis by cellular and/or adenovirus-encoded DNA polymerases, resulting in conversion of the displaced stand into a double stranded form of at least a portion of the DNA molecule. Further replication initiating at the ITR will result in a linear double-stranded DNA molecule, that is flanked by two ITR's, which is larger than the original transfected DNA molecule. A DNA molecule that contains ITR sequences at both ends can replicate itself in transfected cells by virtue of the presence of at least the adenovirus E2 proteins (viz. the DNA-binding protein (DBP), the adenovirus DNA polymerase (Ad-pol), and the preterminal protein (pTP). The required proteins may be expressed from adenovirus genes on the DNA molecule itself, from adenovirus E2 genes integrated in the host-cell genome, or from a replicating helper fragment as described above.

Several groups have shown that the presence of ITR sequences at the end of DNA molecules are sufficient to generate adenovirus minichromosomes that can replicate, if the adenovirus-proteins required for replication are provided in trans e.g. by infection with a helpervirus (Hu et al., 1992); (Wang and Pearson, 1985); (Hay et al., 1984). Hu et *al.*, (1992) observed the presence and replication of symmetrical adenovirus minichromosome-dimers after transfection of plasmids containing a single

ITR. The authors were able to demonstrate that these dimeric minichromosomes arize after tail-to-tail ligation of the single ITR DNA molecules. In DNA extracted from defective adenovirus type 2 particles, dimeric molecules of various sizes have also been observed using electron-microscopy (Daniell, 1976). It was suggested that the incomplete genomes were formed by illegitimate recombination between different molecules and that variations in the position of the sequence at which the illegitimate base pairing occurred were resonsible for the heterogeneous nature of the incomplete genomes. Based on this mechanism it was speculated that, in theory, defective molecules with a total length of up to two times the normal genome could be generated. Such molecules could contain duplicated sequences from either end of the genome. However, no DNA molecules larger than the full-length virus were found packaged in the defective particles (Daniell, 1976). This can be explained by the size-limitations that apply to the packaging. In addition, it was observed that in the virus particles DNA-molecules with a duplicated left-end predominated over those containing the right-end terminus (Daniell, 1976). This is fully explained by the presence of the encapsidation signal near that left-end of the genome (Gräble and Hearing, 1990; Gräble and Hearing, 1992; Hearing et al., 1987).

The major problems associated with the current adenovirus-derived vectors are:

A) The strong immunogenicity of the virus particle

B) The expression of adenovirus genes that reside in the adenoviral vectors, resulting in a Cytotoxic T-cell response against the transduced cells.

C) The low amount of heterologous sequences that can be accommodated in the current vectors (Up to maximally approx. 8000 bp. of heterologous DNA).

Ad A) The strong immunogenicity of the adenovirus particle results in an immunological response of the host, even after a single administration of the adenoviral vector. As a result of the development of neutralizing antibodies, a subsequent administration of the virus will be less effective or even completely ineffective. However, a prolonged or persistent expression of the transferred genes will reduce the number of administrations required and may bypass the problem.

Ad B) Experiments performed by Wilson and collaborators have demonstrated that after adenovirus-mediated gene transfer into immunocompetent animals, the expression of the transgene gradually decreases and disappears approximately 2 - 4 weeks post-infection (Yang et al., 1994a; Yang et al., 1994b). This is caused by the development of a Cytotoxic T-Cell (CTL) response against the transduced cells. The CTLs were directed against adenovirus proteins expressed by the viral vectors. In the transduced cells synthesis of the adenovirus DNA-binding protein (the E2A-gene product), penton and fiber proteins (late-gene products) could be established. These adenovirus proteins, encoded by the viral vector, were expressed despite deletion of the E1 region. This demonstrates that deletion of the E1 region is not sufficient to completely prevent expression of the viral genes (Engelhardt et al., 1994a).

Ad C) Studies by Graham and collaborators have demonstrated that adenoviruses are capable of encapsidating DNA of up to 105% of the normal genome size (Bett et al., 1993). Larger genomes tend to be instable resulting in loss of DNA sequences during propagation of the virus. Combining deletions in the E1 and E3 regions of the virual genomes increases the maximmum size of the foreign that can be encapsidated to approx. 8.3 kb. In addition, some sequences of the E4 region appear to be dispensable for virus growth (adding another 1.8 kb to the maximum encapsidation capacity). Also the E2A region can

be deleted from the vector, when the E2A gene product is provided in trans in the encapsidation cell line, adding another 1.6 kb. It is, however, unlikely that the maximum capacity of foreign DNA can be significantly increased further than 12 kb.

We developed a new strategy for the generation and production of helperfree-stocks of recombinant adenovirus vectors that can accomodate up to 38 kb of foreign DNA. Only two functional ITR sequences, and sequences that can function as an encapsidation signal need to be part of the vector genome. Such vectors are called minimal adenovectors. The helper functions for the minimal adenovectors are provided in trans by encapsidation defective-replication competent DNA molecules that contain all the viral genes encoding the required gene products, with the exception of those genes that are present in the host-cell genome, or genes that reside in the vector genome.

The applications of the disclosed inventions are outlined below and will be illustrated in the experimental part, which is only intended for said purpose, and should not be used to reduce the scope of the present invention as understood by the person skilled in the art.

Use of the IG packaging constructs Diploid cells.

The constructs, in particular pIG.E1A.E1B, will be used to transfect diploid human cells, such as Human Embryonic Retinoblasts (HER), Human Embryonic Kidney cells (HEK), and Human Embryonic Lung cells (HEL). Transfected cells will be selected for transformed phenotype (focus formation) and tested for their ability to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.TK. Such cell lines will be used for the generation and (large-scale) production of E1-deleted recombinant adenoviruses. Such cells, infected with recombinant adenovirus are also intended to be used

in vivo as a local producer of recombinant adenovirus, e.g. for the treatment of solid tumors.

911 cells are used for the titration, generation and production of recombinant adenovirus vectors (Fallaux et al., 1996).

HER cells transfected with pIG.E1A.E1B has resulted in 7 independent clones (called PER cells). These clones are used for the production of E1 deleted (including non-overlapping adenovirus vectors) or E1 defective recombinant adenovirus vectors and provide the basis for introduction of e.g. E2B or E2A constructs (e.g. ts125E2A, see below), E4 etc., that will allow propagation of adenovirus vectors that have mutations in e.g. E2A or E4.

In addition, diploid cells of other species that are permissive for human adenovirus, such as the cotton rat *(Sigmodon hispidus)* (Pacini et al., 1984), Syrian hamster (Morin et al., 1987) or chimpanzee (Levrero et al., 1991), will be immortalized with these constructs. Such cells, infected with recombinant adenovirus, are also intended to be used *in vivo* for the local production of recombinant adenovirus, e.g. for the treatment of solid tumors.

Established cells.

The constructs, in particular pIG.E1A.NEO, can be used to transfect established cells, e.g. A549 (human bronchial carcinoma), KB (oral carcinoma), MRC-5 (human diploid lung cell line) or GLC cell lines (small cell lung cancer) (de Leij et al., 1985; Postmus et al., 1988) and selected for NEO resistance. Individual colonies of resistant cells are isolated and tested for their capacity to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.TK. When propagation of E1 deleted viruses on E1A containing cells is possible, such cells can be used for the generation and production of E1-deleted recombinant adenovirus. They are also be used

for the propagation of E1A deleted/E1B retained recombinant adenovirus.

Established cells can also be co-transfected with pIG.E1A.E1B and pIG.NEO (or another NEO containing expression vector). Clones resistant to G418 are tested for their ability to support propagation of E1 deleted recombinant adenovirus, such as IG.Ad.MLPI.TK and used for the generation and production of E1 deleted recombinant adenovirus and will be applied in vivo for local production of recombinant virus, as described for the diploid cells (see above).

All cell lines, including transformed diploid cell lines or NEO-resistant established lines, can be used as the basis for the generation of 'next generation' packaging cells lines, that support propagation of E1-defective recombinant adenoviruses, that also carry deletions in other genes, such as E2A and E4. Moreover, they will provide the basis for the generation of minimal adenovirus vectors as disclosed herein.

E2 expressing cell lines

Packaging cells expressing E2A sequences are and will be used for the generation and (large scale) production of E2A-deleted recombinant adenovirus.

The newly generated human adenovirus packaging cell lines or cell lines derived from species permissive for human adenovirus (E2A or ts125E2A; E1A + E2A; E1A + E1B + E2A; E1A + E2A/ts125; E1A + E1B + E2A/ts125) or non-permissive cell lines such as monkey cells (hrE2A or hr + ts125E2A; E1A + hrE2A; E1A + E1B + hrE2A; E1A + hrE2A/ts125; E1A + E1B + hrE2A/ts125) are and will be used for the generation and (large scale) production of E2A deleted recombinant adenovirus vectors. In addition, they will be applied *in vivo* for local production of recombinant virus, as described for the diploid cells (see above).

Novel adenovirus vectors.

The newly developed adenovirus vectors harboring an E1 deletion of nt. 459-3510 will be used for gene transfer purposes. These vectors are also the basis for the development of further deleted adenovirus vectors that are mutated for e.g. E2A, E2B or E4. Such vectors will be generated e.g. on the newly developed packaging cell lines described above (see 1-3).

Minimal adenovirus packaging system

We disclose adenovirus packaging constructs (to be used for the packaging of minimal adenovirus vectors) may have the following characteristics:

a. the packaging construct replicates

b. the packaging construct can not be packaged because the packaging signal is deleted

c. the packaging construct contains an internal hairpin-forming sequence (see section 'Experimental; suggested hairpin' see Fig. 15)

d. because of the internal hairpin structure, the packaging construct is duplicated, that is the DNA of the packaging construct becomes twice as long as it was before transfection into the packaging cell (in our sample it duplicates from 35 kb to 70 kb). This duplication also prevents packaging. Note that this duplicated DNA molecule has ITR's at both termini (see e.g. Fig. 13)

e. this duplicated packaging molecule is able to replicate like a 'normal adenovirus' DNA molecule

f. the duplication of the genome is a prerequisite for the production of sufficient levels of adenovirus proteins, required to package the minimal adenovirus vector

g. the packaging construct has no overlapping sequences with the minimal vector or cellular sequences that may lead to generation of RCA by homologous recombination.

This packaging system will be used to produce minimal adenovirus vectors. The advantages of minimal adenovirus vectors e.g. for gene therapy of vaccination purposes, are well known (accommodation of up to 38 kb; gutting of all potentially toxic and immunogenic adenovirus genes).

Adenovirus vectors containing mutations in essential genes (including minimal adenovirus vectors) can also be propagated using this system.

Use of intracellular E2 expressing vectors.

Minimal adenovirus vectors are generated using the helper functions provided in trans by packaging-deficient replicating helper molecules. The adenovirus-derived ITR sequences serve as origins of DNA replication in the presence of at least the E2-gene products. When the E2 gene products are expressed from genes in the vector genome (N.B. the gene(s) must be driven by an E1-independent promoter), the vector genome can replicate in the target cells. This will allow an significantly increased number of template molecules in the target cells, and, as a result an increased expression of the genes of interest encoded by the vector. This is of particular interest for approaches of gene therapy in cancer.

Applications of intracellular amplification of linear DNA fragments.

A similar approach could also be taken if amplification of linear DNA fragments is desired. DNA fragments of known or unknown sequence could be amplified

in cells containing the E2-gene products if at least one ITR sequence is located near or at its terminus. There are no apparent constraints on the size of the fragment. Even fragments much larger than the adenovirus genome (36 kb) should be amplified using this approach. It is thus possible to clone large fragments in mammalian cells without either shuttling the fragment into bacteria (such as E.coli) or use the polymerase chain reaction (P.C.R.). At the end stage of an productive adenovirus infection a single cell can contain over 100,000 copies of the viral genome. In the optimal situation, the linear DNA fragments can be amplified to similar levels. Thus, one should be able to extract more than 5 µg of DNA fragment per 10 million cells (for a 35-kbp fragment). This system can be used to express heterologous proteins (equivalent to the Simian Virus 40-based COS-cell system) for research or for therapeutic purposes. In addition, the system can be used to identify genes in large fragments of DNA. Random DNA fragments may be amplified (after addition of ITRs) and expressed during intracellular amplification. Election or,selection of those cells with the desired phenotype can be used to enrich the fragment of interest and to isolate the gene.

EXPERIMENTAL

Generation of cell lines able to transcomplement E1 defective recombinant adenovirus vectors.

1. 911 cell line

We have generated a cell line that harbors E1 sequences of adenovirus type 5, able to trans-complement E1 deleted recombinant adenovirus (Fallaux et al., 1996). This cell line was obtained by transfection of human diploid human embryonic retinoblasts (HER) with pAd5XhoIC, that contains nt. 80 - 5788 of Ad5; one of the resulting transformants was designated 911. This cell line has been shown to be very useful in the propagation of E1 defective

recombinant adenovirus. It was found to be superior to the 293 cells. Unlike 293 cells, 911 cells lack a fully transformed phenotype, which most likely is the cause of performing better as adenovirus packaging line:

plaque assays can be performed faster (4 - 5 days instead of 8-14 days on 293)

monolayers of 911 cells survive better under agar overlay as required for plaque assays

higher amplification of E1-deleted vectors

In addition, unlike 293 cells that were transfected with sheared adenoviral DNA, 911 cells were transfected using a defined construct. Transfection efficiencies of 911 cells are comparable to those of 293.

New packaging constructs.

Source of adenovirus sequences.

Adenovirus sequences are derived either from pAd5.SalB, containing nt. 80 - 9460 of human adenovirus type 5 (Bernards et al., 1983) or from wild-type Ad5 DNA.

pAd5.SalB was digested with SalI and XhoI and the large fragment was religated and this new clone was named pAd5.X/S.

The pTN construct (constructed by Dr. R. Vogels, IntroGene, The Netherlands) was used as a source for the human PGK promoter and the NEO gene.

Human PGK promoter and NEO^R gene.

Transcription of E1A sequences in the new packaging constructs is driven by the human PGK promoter (Michelson et al., 1983; Singer-Sam et al., 1984), derived from plasmid pTN (gift of R. Vogels), which uses pUC119 (Vieira and Messing, 1987) as a backbone. This plasmid was also used as a source for NEO gene fused to the Hepatitis B Virus (HBV) poly-adenylation signal.

Fusion of PGK promoter to E1 genes (Fig. 1)

In order to replace the E1 sequences of Ad5 (ITR, origin of replication and packaging signal) by heterologous sequences we have amplified E1 sequences (nt.459 to nt. 960) of Ad5 by PCR, using primers Ea1 and Ea2 (see Table I). The resulting PCR product was digested with ClaI and ligated into Bluescript (Stratagene), predigested with ClaI and EcoRV, resulting in construct pBS.PCRI.

Vector pTN was digested with restriction enzymes EcoRI (partially) and ScaI, and the DNA fragment containing the PGK promoter sequences was ligated into PBS.PCRI digested with ScaI and EcoRI. The resulting construct PBS.PGK.PCRI contains the human PGK promoter operatively linked to Ad5 E1 sequences from nt.459 to nt. 916.

Construction of pIG.E1A.E1B.X (Fig. 2)

pIG.E1A.E1B.X was made by replacing the ScaI-BspEI fragment of pAT-X/S by the corresponding fragment from PBS.PGK.PCRI (containing the PGK promoter linked to E1A sequences).

pIG.E1A.E1B.X contains the E1A and E1B coding sequences under the direction of the PGK promoter.

As Ad5 sequences from nt.459 to nt. 5788 are present in this construct, also pIX protein of adenovirus is encoded by this plasmid.

Construction of pIG.E1A.NEO (Fig. 3)

In order to introduce the complete E1B promoter and to fuse this promoter in such a way that the AUG codon of E1B 21 kD exactly functions as the AUG codon of NEO^R, we amplified the E1B promoter using primers Ea3 and Ep2, where primer Ep2 introduces an NcoI site in the PCR

fragment. The resulting PCR fragment, named PCRII, was digested with HpaI and NcoI and ligated into pAT-X/S, which was predigested with HpaI and with NcoI. The resulting plasmid was designated pAT-X/S-PCR2. The NcoI - StuI fragment of pTN, containing the NEO gene and part of the Hepatitis B Virus (HBV) poly-adenylation signal, was cloned into pAT-X/S-PCR2 (digested with NcoI and NruI). The resulting construct: pAT-PCR2-NEO. The poly-adenylation signal was completed by replacing the ScaI-SalI fragment of pAT-PCR2-NEO by the corresponding fragment of pTN (resulting in pAT.PCR2.NEO.p(A)). The ScaI - XbaI of pAT.PCR2.NEO.p (A) was replaced by the corresponding fragment of pIG.E1A.E1B-X, containing the PGK promoter linked to E1A genes.

The resulting construct was named pIG.E1A.NEO, and thus contains Ad5 E1 sequences (nt.459 to nt 1713) under the control of the human PGK promoter.

Construction of pIG.EIA.E1B (Fig. 4)

pIG.E1A.E1B was made by amplifying the sequences encoding the N-terminal amino acids of E1B 55kd using primers Eb1 and Eb2 (introduces a XhoI site). The resulting PCR fragment was digested with BglII and cloned into BglII/NruI of pAT-X/S, thereby obtaining pAT-PCR3.

pIG.E1A.E1B was constructed by introducing the HBV poly(A) sequences of pIG.E1A.NEO downstream of E1B sequences of pAT-PCR3 by exchange of XbaI - SalI fragment of pIg.E1A.NEO and the XbaI XhoI fragment of pAT.PCR3.

pIG.E1A.E1B contains nt. 459 to nt. 3510 of Ad5, that encode the E1A and E1B proteins. The E1B sequences are terminated at the splice acceptor at nt.3511. No pIX sequences are present in this construct.

Construction of pIG.NEO (Fig. 5)

pIG.NEO was generated by cloning the HpaI - ScaI fragment of pIG.E1A.NEO, containing the NEO gene under the control of the Ad.5 E1B promoter, into pBS digested with EcoRV and ScaI.

This construct is of use when established cells are transfected with E1A.E1B constructs and NEO selection is required. Because NEO expression is directed by the E1B promoter, NEO resistant cells are expected to co-express E1A, which also is advantageous for maintaining high levels of expression of E1A during long-term culture of the cells.

Testing of constructs.

The integrity of the constructs pIG.E1A.NEO, pIG.E1A.E1B.X and pIG.E1A.E1B was assessed by restriction enzyme mapping; furthermore, parts of the constructs that were obtained by PCR analysis were confirmed by sequence analysis. No changes in the nucleotide sequence were found.

The constructs were transfected into primary BRK (Baby Rat Kidney) cells and tested for their ability to immortalize (pIG.E1A.NEO) or fully transform (pAd5.XhoIC,pIG.E1A.E1B.X and pIG.E1A.E1B) these cells.

Kidneys of 6-day old WAG-Rij rats were isolated, homogenized and trypsinized. Subconfluent dishes (diameter 5 cm) of the BRK cell cultures were transfected with 1 or 5 µg of pIG.NEO, pIG.E1A.NEO, pIG.E1A.E1B, pIG.E1A.E1B.X, pAd5XhoIC, or with pIG.E1A.NEO together with PDC26 (Van der Elsen et al., 1983), carrying the Ad5.E1B gene under control of the SV40 early promoter. Three weeks post-transfection, when foci were visible, the dishes were fixed, Giemsa stained and the foci counted.

An overview of the generated adenovirus packaging constructs, and their ability to transform BRK, is

presented in Fig. 6. The results indicate that the constructs pIG.E1A.E1B and pIG.E1A.E1B.X are able to transform BRK cells in a dose-dependent manner. The efficiency of transformation is similar for both constructs and is comparable to what was found with the construct that was used to make 911 cells, namely pAd5.XhoIC.

As expected, pIG.E1A.NEO was hardly able to immortalize BRK. However, co-transfection of an E1B expression construct (PDC26) did result in a significant increase of the number of transformants (18 versus 1), indicating that E1A encoded by pIG.E1A.NEO is functional.

We conclude therefore, that the newly generated packaging constructs are suited for the generation of new adenovirus packaging lines.

Generation of cell lines with new packaging constructs Cell lines and cell culture

Human A549 bronchial carcinoma cells (Shapiro et al., 1978), human embryonic retinoblasts (HER), Ad5-E1-transfor-med human embryonic kidney (HEK) cells (293; Graham et al., 1977) cells and Ad5-transformed HER cells (911; Fallaux et al, 1996)) and PER cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics in a 5% C02 atmosphere at 37°C. Cell culture media, reagents and sera were purchased from Gibco Laboratories (Grand Island, NY). Culture plastics were purchased from Greiner (Nürtingen, Germany) and Corning (Corning, NY).

Viruses and virus techniques

The construction of adenoviral vectors IG.Ad.MLP.nls.lacZ, IG.Ad.MLP.luc, IG.Ad.MLP.TK and IG.Ad.CMV.TK is described in detail in patent application EP 95202213.

The recombinant adenoviral vector IG.Ad.MLP.nls.lacZ contains the E.coli lacZ gene, encoding β-galactosidase, under control of the Ad2 major late promoter (MLP).IG.Ad.MLP.luc contains the firefly luciferase gene driven by the Ad2 MLP. Adenoviral vectors IG.Ad.MLP.TK and IG.Ad.CMV.TK contain the Herpes Simplex Virus thymidine kinase (TK) gene under the control of the Ad2 MLP and the Cytomegalovirus (CMV) enhancer/promoter, respectively.

Transfections

All transfections were performed by calcium-phosphate precipitation DNA (Graham and Van der Eb, 1973) with the GIBCO Calcium Phosphate Transfection System (GIBCO BRL Life Technologies Inc., Gaithersburg, USA), according to the manufacturers protocol.

Western blotting

Subconfluent cultures of exponentially growing 293,911 and Ad5-E1-transformed A549 and PER cells were washed with PBS and scraped in Fos-RIPA buffer (10 mM Tris (pH 7,5), 150 mM NaCl, 1% NP40,01% sodium dodecyl sulphate (SDS), 1% NA-DOC, 0,5 mM phenyl methyl sulphonyl fluoride (PMSF), 0,5 mM trypsin inhibitor, 50 mM NaF and 1 mM sodium vanadate). After 10 min. at room temperature, lysates were cleared by centrifugation. Protein concentrations were measured with the Biorad protein assay kit, and 25 µg total cellular protein was loaded on a 12.5% SDS-PAA gel. After electrophoresis, proteins were transferred to nitrocellulose (1h at 300 mA). Prestained standards (Sigma, USA) were run in parallel. Filters were blocked with 1% bovine serum albumin (BSA) in TBST (10 mM Tris, pH 8, 15 mM NaCl, and 0.05% Tween-20) for 1 hour. First antibodies were the mouse monoclonal anti-Ad5-E1B-55-kDA antibody A1C6 (Zantema et al., unpublished), the rat monoclonal anti-Ad5-E1B-221-kDa antibody C1G11

(Zantema et al., 1985). The second antibody was a horseradish peroxidase-labeled goat anti-mouse antibody (Promega). Signals were visualized by enhanced chemoluminescence (Amersham Corp, UK).

Southern blot analysis

High molecular weight DNA was isolated and 10 µg was digested to completion and fractionated on a 0.7% agarose gel. Southern blot transfer to Hybond N+ (Amersham, UK) was performed with a 0.4 M NAOH, 0.6 M NaCl transfer solution (Church and Gilbert, 1984). Hybridization was performed with a 2463-nt SspI-HindIII fragment from pAd5.SalB (Bernards et al., 1983). This fragment consists of Ad5 bp. 342-2805. The fragment was radiolabeled with α-^{32}p-dCTP with the use of random hexanucleotide primers and Klenow DNA polymerase. The southern blots were exposed to a Kodak XAR-5 film at -80°C and to a Phospho-Imager screen which was analyzed by B&L systems Molecular Dynamics software.

A549

Ad5-E1-transformed A549 human bronchial carcinoma cell lines were generated by transfection with pIG.E1A.NEO and selection for G418 resistance. Thirty-one G418 resistant clones were established. Co-transfection of pIG.E1A.E1B with pIG.NEO yielded seven G418 resistant cell lines.

PER

Ad5-E1-transformed human embryonic retina (HER) cells were generated by transfection of primery HER cells with plasmid pIG.E1A.E1B. Transformed cell lines were established from well-separated foci. We were able to

establish seven clonal cell lines, which we called PER.C1, PER.C3, PER.C4, PER.C5, PER.C6, PER.C8 and PER.C9.

One of the PER clones, namely PER.C6, has been deposited at the ECACC under number 96022940.

Expression of Ad5 E1A and E1B genes in transformed A549 and PER cells

Expression of the Ad5 E1A and the 55-kDa and 21 kDa E1B proteins in the established A549 and PER cells was studied by means of Western blotting, with the use of monoclonal antibodies (mAb). Mab M73 recognizes the E1A products, whereas Mabls AIC6 and ClGll are directed against the 55-kDa and 21 kDa E1B proteins, respectively.

The antibodies did not recognize proteins in extracts from the parental A549 or the primary HER cells (data not shown). None of the A549 clones that were generated by co-transfection of pIG.NEO and pIG.E1A.E1B expressed detectable levels of E1A or E1B proteins (not shown). Some of the A549 clones that were generated by transfection with pIG.E1A.NEO expressed the Ad5 E1A proteins (Fig. 7), but the levels were much lower than those detected in protein lysates from 293 cells. The steady state E1A levels detected in protein extracts from PER cells were much higher than those detected in extracts from A549-derived cells. All PER cell lines expressed similar levels of E1A proteins (Fig. 7). The expression of the E1B proteins, particularly in the case of E1B 55 kDa, was mote variable. Compared to 911 and 293, the majority of the PER clones express high levels of E1B 55 kDa and 21 kDa. The steady state level of E1B 21 kDa was the highest in PER.C3. None of the PER clones lost expression of the Ad5 El genes upon serial passage of the cells (not shown). We found that the level of El expression in PER cells remained stable for at least 100 population doublings. We decided to characterize the PER clones in more detail.

Southern analysis of PER clones

To study the arrangement of the Ad5-E1 encoding sequences in the PER clones we performed Southern analyses. Cellular DNA was extracted from all PER clones, and from 293 and 911 cells. The DNA was digested with HindIII, which cuts once in the Ad5 E1 region. Southern hybridization on HindIII-digested DNA, using a radiolabeled Ad5-E1-specific probe revealed the presence of several integrated copies of pIG.E1A.E1B in the genome of the PER clones. Figure 8 shows the distribution pattern of E1 sequences in the high molecular weight DNA of the different PER cell lines. The copies are concentrated in a single band, which suggests that they are integrated as tandem repeats. In the case of PER.C3, C5, C6 and C9 we found additional hybridizing bands of low molecular weight that indicate the presence of truncated copies of pIG.E1A.E1B. The number of copies was determined with the use of a Phospho-Imager. We estimated that PER.C1, C3, C4, C5, C6, C8 and C9 contain 2, 88, 5,4, 5, 5 and 3 copies of the Ad5 E1 coding region, respectively, and that 911 and 293 cells contain 1 and 4 copies of the Ad5 E1 sequences, respectively.

Transfection efficiency

Recombinant adenovectors are generated by co-transfection of adaptor plasmids and the large ClaI fragment of Ad5 into 293 cells (gee patent application EP 95202213). The recombinant virus DNA is formed by homologous recombination between the homologous viral sequences that are present in the plasmid and the adenovirus DNA. The efficacy of this method, as well as that of alternative strategies, is highly dependent on the transfectability of the helper cells. Therefore, we compared the transfection efficiencies of some of the

PER clones with 911 cells, using the E.coli β-galactosidase-encoding lacZ gene as a reporter (Fig. 9).

Production of recombinant adenovirus

Yields of recombinant adenovirus obtained after inoculation of 293, 911, PER.C3, PER.C5 and PER.C6 with different adenovirus vectors are presented in Table II.

The results indicate that the yields obtained on PER cells are at least as high as those obtained on the existing cell lines.

In addition, the yields of the novel adenovirus vector IG.Ad.MLPI.TK are similar or higher than the yields obtained for the other viral vectors on all cell lines tested.

Generation of new adenovirus vectors (Fig. 10).

The used recombinant adenovirus vectors (see patent application on EP 95202213) are deleted for E1 sequences from 459 to nt. 3328.

As construct pE1A.E1B contains Ad5 sequences 459 to nt. 3510 there is a sequence overlap of 183 nt. between E1B sequences in the packaging construct pIG.E1A.E1B and recombinant adenoviruses, such as e.g. IG.Ad.MLP.TK. The overlapping sequences were deleted from the new adenovirus vectors. In addition, non-coding sequences derived from lacZ, that are present in the original contructs, were deleted as well. This was achieved (see Fig. 10) by PCR amplification of the SV40 poly(A) sequences from pMLP.TK using primers SV40-1 (introduces a BamHI site) and SV40-2 (introduces a BglII site). In addition, Ad5 sequences present in this construct were amplified from nt 2496 (Ad5, introduces a BglII site) to nt. 2779 (Ad5-2). Both PCR fragments were digested with BglII and were ligated. The ligation product was PCR amplified using primers SV40-1 and Ad5-2. The PCR product obtained was cut with

BamHI and AflII and was ligated into pMLP.TK predigested with the same enzymes. The resulting construct, named pMLPI.TK, contains a deletion in adenovirus E1 sequences from nt 459 to nt. 3510.

Packaging system

The combination of the new packaging construct pIG.E1A.E1B and the recombinant adenovirus pMLPI.TK, which do not have any sequence overlap, are presented in Fig. 11. In this figure, also the original situation is presented, where the sequence overlap is indicated.

The absence of overlapping sequences between pIG.E1A.E1B and pMLPI.TK (Fig. 11a) excludes the possibility of homologous recombination between packaging construct and recombinant virus, and is therefore a significant improvement for production of recombinant adenovirus as compared to the original situation.

In Fig. 11b the situation is depicted for pIG.E1A.NEO and IG.Ad.MLPI.TK. pIG.E1A.NEO when transfected into established cells, is expected to be sufficient to support propagation of E1-deleted recombinant adenovirus. This combination does not have any sequence overlap, preventing generation of RCA by homologous recombination. In addition, this convenient packaging system allows the propagation of recombinant adenoviruses that are deleted just for E1A sequences and not for E1B sequences. Recombinant adenoviruses expressing E1B in the absence of E1A are attractive, as the E1B protein, in particular E1B 19kD, is able to prevent infected human cells from lysis by Tumor Necrosis Factor (TNF) (Gooding et al., 1991).

Generation of recombinant adenovirus derived from pMLPI.TK.

Recombinant adenovirus was generated by co-transfection of 293 cells with SalI linearized pMLPI.TK

DNA and ClaI linearized Ad5 wt DNA. The procedure is schematically represented in Fig. 12.

Outline of the strategy to generate packaging systems for minimal adenovirus vector

Name convention of the plasmids used:

p plasmid
I ITR (Adenovirus Inverted Terminal Repeat)
C Cytomegalovirus (CMV) Enhancer/Promoter Combination
L Firefly Luciferase Coding Sequence hac,haw Potential hairpin that can be formed after digestion with restriction endonuclease Asp718 in its correct and in the. reverse orientation, respectively (Fig. 15).

Eg. pICLhaw is a plasmid that contains the adenovirus ITR followed by the CMV-driven luciferase gene and the Asp718 hairpin in the reverse (non-functional) orientation.

1.1 Demonstration of the competence of a synthetic DNA sequence, that is capable of forming a hairpin-structure, to serve as a primer for reverse strand synthesis for the generation of double-stranded DNA molecules in cells that contain and express adenovirus genes.
Plasmids pICLhac, pICLhaw, pICLI and pICL were generated using standard techniques. The schematic representation of these plasmids is shown in Figs. 16-19.
Plasmid pICL is derived from the following plasmids:
nt.1 - 457 pMLP10 (Levrero et al., 1991)
nt.458 - 1218 pCMVβ (Clontech, EMBL Bank No. U02451)
nt.1219 - 3016 pMLP.luc (IntroGene, unpublished)
nt.3017 - 5620 pBLCAT5 (Stein and Whelan, 1989)

The plasmid has been constructed as follows:

The *tet* gene of plasmid pMLP10 has been inactivated by deletion of the BamHI-SalI fragment, to generate pMLP10ΔSB. Using primer set PCR/MLP1 and PCR/MLP3 a 210 bp fragment containing the Ad5-ITR, flanked by a synthetic SalI restriction site was amplified using pMLP10 DNA as the template. The PCR product was digested with the enzymes EcoRI and SgrAI to generate a 196 bp. fragment. Plasmid pMLP10ΔSB was digested with EcoRI and SgrAI to remove the ITR. This fragment was replaced by the EcoRI-SgrAI-treated PCR fragment to generate pMLP/SAL. Plasmid pCMV-Luc was digested with PvuII to completion and recirculated to remove the SV40-derived poly-adenylation signal and Ad5 sequences with exception of the Ad5 left-terminus. In the resulting plasmid, pCMV-lucΔAd, the Ad5 ITR was replaced by the Sal-site-flanked ITR from plasmid pMLP/SAL by exchanging the XmnI-SacII fragments. The resulting plasmid, pCMV-lucΔAd/SAL, the Ad5 left terminus and the CMV-driven luciferase gene were isolated as an SalI-SmaI fragment and inserted in the SalI and HpaI digested plasmid pBLCATS, to form plasmid pICL. Plasmid pICL is represented in Fig 19; its sequence is presented in Fig. 20.

Plasmid pICL contains the following features:

nt. 1-457	Ad5 left terminus (Sequence 1-457 of human adenovirus type 5)
nt. 458-969	Human cytomegalovirus enhancer and immediate
early promoter	(Boshart et al., 1985)(from plasmid pCMVβ, Clontech, Palo Alto, USA)
nt. 970-1204	SV40 19S exon and truncated 16/19S intron
	(from plasmid pCMVβ)
nt. 1218-2987	Firefly luciferase gene (from pMLP.luc)

nt. 3018-3131	SV40 tandem poly-adenylation signals from late transcript, derived from plasmid pBLCAT5)
nt. 3132-5620	pUC12 backbone (derived from plasmid pBLCAT5)
nt. 4337-5191	β-lactamase gene (Amp-resistence gene, reverse orientation)

Plasmid pICLhac and pICLhaw

Plasmids pICLhac and pICLhaw were derived from plasmid pICL by digestion of the latter plasmid with the restriction enzyme Asp718. The linearized plasmid was treated with Calf-Intestine Alkaline Phosphatase to remove the 51 phoshate groups. The partially complementary synthetic single-stranded oligonucleotide Hp/aspl en Hp/asp2 were annealed and phosphorylated on their 5'ends using T4-polynucleotide kinase.

The phosporylated double-stranded oligomers were mixed with the dephosporylated pICL fragment and ligated. Clones containing a single copy of the synthetic oligonucleotide inserted into the plasmid were isolated and characterized using restriction enzyme digests. Insertion of the oligonucleotide into the Asp718 site will at one junction recreate an Asp718 recognition site, whereas at the other junction the recognitionsite will be disrupted. The orientation and the integrity of the inserted oligonucleotide was verified in selected clones by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the Asp718 site close to the 3205 EcoRI site) was denoted pICLhac. A clone with the oligonucleotide in the reverse orientation (the Asp718 site close to the SV40 derived poly signal) was designated pICLhaw. Plasmids pICLhac and pICLhaw are represented in Figs. 16 and 17.

Plasmid pICLI was created from plasmid pICL by insertion of the SalI-SgrAI fragment from pICL, containing

the Ad5-ITR into the Asp718 site of pICL. The 194 bp SalI-SgrAI fragment was isolated from pICL, and the cohesive ends were converted to blunt ends using E.coli DNA polymerase I (Klenow fragment) and dNTP's. The Asp718 cohesive ends were converted to blunt ends by treatment with mungbean nuclease. By ligation clones were generated that contain the ITR in the Asp718 site of plasmid pICL. A clone that contained the ITR fragment in the correct orientation was designated pICLI (Fig. 18).

Generation of adenovirus Ad-CMV-hcTK. Recombinant adenovirus was constructed according to the method described in Patent application 95202213. Two components are required to generate a recombinant adenovirus. First, an adaptor-plasmid containing the left terminus of the adenovirus genome containing the ITR and the packaging signal, an expression cassette with the gene of interest, and a portion of the adenovirus genome which can be used for homologous recombination. In addition, adenovirus DNA is needed for recombination with the aforementioned adaptor plasmid. In the case of Ad-CMV-hcTK, the plasmid PCMV.TK was used as a basis. This plasmid contains nt. 1-455 of the adenovirus type 5 genome, nt. 456-1204 derived from pCMVβ (Clontech, the PstI-StuI fragment that contains the CMV enhancer promoter and the 16S/19S intron from Simian Virus 40), the Herpes Simplex Virus thymidine kinase gene (described in Patent application 95202213), the SV40-derived polyadenylation signal (nt. 2533-2668 of the SV40 sequence), followed by the BglII-ScaI fragment of Ad5 (nt. 3328-6092 of the Ad5 sequence). These fragments are present in a pMLP10-derived (Levrero et al., 1991) backbone. To generate plasmid pAD-CMVhc-TK, plasmid pCMV.TK was digested with ClaI (the unique ClaI-site is located just upstream of the TK open readingframe) and dephosphorylated with Calf-Intestine Alkaline Phosphate. To generate a hairpin-structure, the synthetic oligonucleotides HP/cla2 and HP/cla2 were annealed and phopsphorylated on their 5'-OH groups with T4-

polynucleotide kinase and ATP. The double-stranded oligonucleotide was ligated with the linearized vector fragment and used to transform E.coli strain "Sure". Insetion of the oligonucleotide into the ClaI site will disrupt the ClaI recognition sites. In the oligonucleotide contains a new ClaI site near one of its termini. In selected clones, the orientation and the inegrity of the inserted oligonucleotide was verified by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the ClaI site at the ITR side) was denoted pAd-CMV-hcTK. This plasmid was co-transfected with ClaI digested wild-type Adenovirus-type5 DNA into 911 cells. A recombinant adenovirus in which the CMV-hcTK expression cassette replaces the El sequences was isolated and propagated using standard procedures.

To study whether the hairpin can be used as a primer for reverse strand synthesis on the displaced strand after replication had started at the ITR, the plasmid pICLhac is introduced into 911 cells (human embryonic retinoblasts transformed with the adenovirus El region). The plasmid pICLhaw serves as a control, which contains the oligonucleotide pair HP/asp 1 and 2 in the reverse orientation but is further completely identical to plasmid pICLhac. Also included in these studies are plasmids pICLI and pICL. In the plasmid pICLI the hairpin is replaced by an adenovirus ITR. Plasmid pICL contains neither a hairpin nor an ITR sequence. These plasmids serve as controls to determine the efficiency of replication by virtue of the terminal-hairpin structure. To provide the viral products other than the El proteins (these are produced by the 911 cells) required for DNA replication the cultures are infected with the virus IG.Ad.MLPI.TK after transfection. Several parameters are being studied to demonstrate proper replication of the transfected DNA molecules. First, DNA extracted from the cell cultures transfected with aforementioned plasmids and infected with IG.Ad.MLPI.TK virus is being analyzed by Southern blotting for the

presence of the expected replication intermediates, as well as for the presence of the duplicated genomes. Furthermore, from the transfected and IG.Ad.MLPI.TK infected cell populations virus is isolated, that is capable to transfer and express a luciferase marker gene into luciferase negative cells.

Plasmid DNA of plasmids pICLhac, pICLhaw, pICLI and pICL have been digested with restriction endonuclease SalI and treated with mungbean nuclease to remove the 4 nucleotide single-stranded extension of the resulting DNA fragment. In this manner a natural adenovirus 5'ITR terminus on the DNA fragment is created. Subsequently, both the pICLhac and pICLhaw plasmids were digested with restriction endonuclease Asp718 to generate the terminus capable of forming a hairpin structure. The digested plasmids are introduced into 911 cells, using the standard calcium phosphate co-precipitation technique, four dishes for each plasmid. During the transfection, for each plasmid two of the cultures are infected with the IG.Ad.MLPI.TK virus using 5 infectious IG.Ad.MLPI.TK particles per cell. At twenty-hours post-transfection and fort hours post-transfection one Ad.tk-virus-infected and one uninfected culture are used to isolate small molecular-weight DNA using the procedure devised by Hirt. Aliquots of isolated DNA are used for Southern analysis. After digestion of the samples with restriction endonuclease EcoRI using the luciferase gene as a probe a hybridizing fragment of approx. 2.6kb is detected only in the samples from the adenovirus infected cells transfected with plasmid pICLhac. The size of this fragment is consistent with the anticipated duplication of the luciferase marker gene. This supports the conclusions that the inserted hairpin is capable to serve as a primer for reverse strand synthesis. The hybridizing fragment is absent if the IG.Ad.MLPI.TK virus is omitted, or if the hairpin oligonucleotide has been inserted in the reverse orientation.

The restriction endonuclease DpnI recognizes the tetranucleotide sequence 5'-GATC-3', but cleaves only methylated DNA, (that is, only (plasmid) DNA propagated in, and derived, from E.coli, not DNA that has been replicated in mammalian cells). The restriction endonuclease MboI recognizes the same sequences, but cleaves only unmethylated DNA (viz. DNA propagated in mammalian cells). DNA samples isolated from the transfected cells are incubated with MboI and DpnI and analysed with Southern blots. These results demonstrate that only in the cells transfected with the pICLhac and the pICLI plasmids large DpnI-resistant fragments are present, that are absent in the MboI treated samples. These data demonstrate that only after transfection of plasmids pICLI and pICLhac replication and duplication of the fragments occur.

These data demonstrate that in ~adenovirus-infected cells linear DNA fragments that have on one terminus an adenovirus-derived inverted terminal repeat (ITR) and at the other terminus a nucleotide sequence that can anneal to sequences on the same strand, when present in single-stranded form thereby generate a hairpin structure, and will be converted to structures that have inverted terminal repeat sequences on both ends. The resulting DNA molecules will replicate by the same mechanism as the wild type adenovirus genomes.

1.2 Demonstration that the DNA molecules that contain a luciferase marker gene, a single copy of the ITR, the encapsidation signal and a synthetic DNA sequence, that is capable of forming a hairpin structure, are sufficient to generate DNA molecules that can be encapsidated into virions.

To demonstrate that the above DNA molecules containing two copies of the CMV-luc marker gene can be encapsidated into virions, virus is harvested from the remaining two cultures via three cycles of freeze-thaw

crushing and is used to infect murine fibroblasts. Forty-eight hours after infection the infected cells are assayed for luciferase activity. To exclude the possibility that the luciferase activity has been induced by transfer of free DNA, rather than via virus particles, virus stocks are treated with DNaseI to remove DNA contaminants. Furthermore, as an additional control, aliquots of the virus stocks are incubated for 60 minutes at 56°C. The heat treatment will not affect the contaminating DNA, but will inactivate the viruses. Significant luciferase activity is only found in the cells after infection with the virus stocks derived from IG.Ad.MLPI.TK-infected cells transfected with the pICLhc and pICLI plasmids. Neither in the non-infected cells, nor in the infected cells transfected with the pICLhw and pICL significant luciferase activity can be demonstrated. Heat inactivation, but not DNaseI treatment, completely eliminates luciferase expression, demonstrating that adenovirus particles, and not free (contaminating) DNA fragments are responsible for transfer of the luciferase reporter gene.

These results demonstrate that these small viral genomes can be encapsidated into adenovirus particles and suggest that the ITR and the encapsidation signal are sufficient for encapsidation of linear DNA fragments into adenovirus particles. These adenovirus particles can be used for efficient gene transfer. When introduced into cells that contain and express at least part of the adenovirus genes (viz. E1, E2, E4, and L, and VA), recombinant DNA molecules that consist of at least one ITR, at least part of the encapsidation signal as well as a synthetic DNA sequence, that is capable of forming a hairpin structure, have the intrinsic capacity to autonomously generate recombinant genomes which can be encapsidated into virions. Such genomes and vector system can be used for gene transfer.

1.3 Demonstration that DNA molecules which contain nucleotides 3510 - 35953 (viz. 9.7 - 100 map units) of the adenovirus type 5 genome (thus lack the El protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion of the same strand of the DNA molecule when present in single-stranded form other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells.

In order to develop a replicating DNA molecule that can provide the adenovirus products required to allow the above mentioned ICLhac vector genome and alike minimal adenovectors to be encapsidated into adenovirus particles by helper cells, the Ad-CMV-hcTK adenoviral vector has been developed. Between the CMV enhancer/promoter region and the thymidine kinase gene the annealed oligonucleotide pair HP/cla 1 and 2 is inserted. The vector Ad-CMV-hcTK can be propagated and produced in 911 cell using standard procedures. This vector is grown and propagated exclusively as a source of DNA used for transfection. DNA of the adenovirus Ad-CMV-hcTK is isolated from virus particles that had been purified using CsCl density-gradient centrifugation by standard techniques. The virus DNA has been digested with restriction endonuclease ClaI. The digested DNA is size-fractionated on an 0.7% agarose gel and the large fragment is isolated and used for further experiments. Cultures of 911 cells are transfected large ClaI-fragment of the Ad-CMV-hcTK DNA using the standard calcium phosphate co-precipitation technique. Much like in the previous experiments with plasmid pICLhac, the AD-CMV-hc will replicate starting at the right-hand ITR. Once the 1-strand is displaced, a hairpin can be formed at the left-hand terminus of the fragment. This facilitates the DNA polymerase to elongate the chain towards the right-hand-side. The process will proceed until the displaced strand is completely converted to its double-stranded form. Finally, the right-hand ITR will be

recreated, and in this location the normal adenovirus replication-initiation and elongation will occur. Note that the polymerase will read through the hairpin, thereby duplicating the molecule. The input DNA molecule of 33250 bp, that had on one side an adenovirus ITR sequence and at the other side a DNA sequence that had the capacity to form a hairpin structure, has now been duplicated, in a way that both ends contain an ITR sequence. The resulting DNA molecule will consist of a palindromic structure of approximately 66500 bp.

This structure can be detected in low-molecular weight DNA extracted from the transfected cells using Southern analysis. The palindromic nature of the DNA fragment can be demonstrated by digestion of the low-molecular weight DNA with suitable restriction endonucleases and Southern blotting with the HSV-TK gene as the probe. This molecule can replicate itself in the transfected cells by virtue of the adenovirus gene products that are present in the cells. In part, the adenovirus genes are expressed from templates that are integrated in the genome of the target cells (viz. the E1 gene products), the other genes reside in the replicating DNA fragment itself. Note however, that this linear DNA fragment cannot be encapsidated into virions. Not only does it lack all the DNA sequences required for encapsidation, but also is its size much too large to be endapsidated.

1.4 Demonstration that DNA molecules which contain nucleotides 3503 - 35953 (viz. 9.7 - 100 map units) of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion the same strand of the DNA molecule other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells and can provide the helper functions required to encapsidate the pICLI and pICLhac derived DNA fragments.

The next series of experiments aim to demonstrate that the DNA molecule described in part 1.3 could be used to encapsidate the minimal adenovectors described in part 1.1 and 1.2.

In the experiments the large fragment isolated after endonuclease ClaI-digestion of Ad-CMV-hcTK DNA is introduced into 911 cells (conform the experiments described in part 1.3) together with endonuclease SalI, mungbean nuclease, endonuclease Asp718-treated plasmid pICLhac, or as a control similarly treated plasmid pICLhaw. After 48 hours virus is isolated by freeze-thaw crushing of the transfected cell population. The virus-preparation is treated with DNaseI to remove contaminating free DNA. The virus is used subsequently to infect Rat2 fibroblasts. Forty-eight hours post infection the cells are assayed for luciferase activity. Only in the cells infected with virus isolated from the cells transfected with the pICLhac plasmid, and not with the pICLhaw plasmid, significant luciferase activity can be demonstrated. Heatinactivation of the virus prior to infection completely abolishes the luciferase activity, indicating that the luciferase gene is transferred by a viral particle. Infection of 911 cell with the virus stock did not result in any cytopathological effects, demonstrating that the pICLhac is produced without any infectious helper virus that can be propagated on 911 cells. These results demonstrate that the proposed method can be used to produce stocks of minimal-adenoviral vectors, that are completely devoid of infectious helper viruses that are able to replicate autonomously on adenovirus-transformed human cells or on non-adenovirus transformed human cells.

Besides the system described in this application, another approach for the generation of minimal adenovirus vectors has been disclosed in WO 94/12649. The method described in WO 94/12649 exploits the function of the protein IX for the packaging of minimal adenovirus vectors

(Pseudo Adenoviral Vectors (PAV) in the terminology of WO 94/12649). PAVs are produced by cloning an expression plasmid with the gene of interest between the left-hand (including the sequences required for encapsidation) and the right-hand adenoviral ITRs. The PAV is propagated in the presence of a helper virus. Encapsidation of the PAV is preferred compared the helper virus because the helper virus is partially defective for packaging. (Either by virtue of mutations in the packaging signal or by virtue of its size (virus genomes greater than 37.5 kb package inefficiently). In addition, the authors propose that in the absence of the protein IX gene the PAV will be preferentially packaged. However, neither of these mechanisms appear to be sufficiently restrictive to allow packaging of only PAVs/minimal vectors. The mutations proposed in the packaging signal diminish packaging, but do not provide an absolute block as the same packaging-activity is required to propagate the helper virus. Also neither an increase in the size of the helper virus nor the mutation of the protein IX gene will ensure that PAV is packaged exclusively. Thus, the method described in WO 94/12649 is unlikely to be useful for the production of helper-free stocks of minimal adenovirus vectors/PAVs.

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Table I

Primers used for PCR amplification of DNA fragments used for generation of constructs described in this patent application.

Ea-1	CGTGTAGTGTATTTATACCCG	PCR amplification Ad5 nt459 ->
Ea-2	TCGTCACTGGGTGGAAAGCCA	PCR amplification Ad5 nt960 <-
Ea-3	TACCCGCCGTCCTAAAATGGC	nt1284-1304 of Ad5 genome
Ea-5	TGGACTTGAGCTGTAAACGC	nt1514-1533 of Ad5 genome
Ep-2	GCCTCCATGGAGGTCAGATGT	nt1721-1702 of Ad5; introduction of NcoI site
Eb-1	GCTTGAGCCCGAGACATGTC	nt3269-3289 of Ad5 genome
Eb-2	CCCCTCGAGCTCAATCTGTATCTT	nt3508-3496 of Ad5 genome; introduction of XhoI site
SV40-1	GGGGGATCCGAACTTGTTTATTGCAGC	Introduction BamHI site (nt2182-2199 of pMLP.TK) adaption of ecombinant adenoviruses
SV40-2	GGGAGATCTAGACATGATAAGATAC	Introduction BglII site (nt2312-2297 of pMLP.TK)
Ad5-1	GGGAGATCTGTACTGAAATGTGTGGGC	Introduction BglII site (nt2496-2514 of pMLP.TK)
Ad5-2	GGAGGCTGCAGTCTCCAACGGCGT	nt2779-2756 of PMLP.TK
ITR1	GGGGGATCCTCAAATCGTCACTTCCGT	nt35737-35757 of Ad5 (introduction of BamHI site)
ITR2	GGGGTCTAGACATCATCAATAATATAC	nt35935-35919 of Ad5 (introduction of XbaI site)

PCR primers sets to be used to create the SalI and Asp718 sites juxtaposed to the ITR sequences.

PCR/MLP1	GGCGAATTCGTCGACATCATCAATAATATACC	(Ad5 nt. 10-18)
PCR/MLP2	GGCGAATTCGGTACCATCATCAATAATATACC	(Ad5 nt. 10-18)
PCR/MLP3	CTGTGTACACCGGCGCA	(Ad5 nt.200-184)

Synthetic oligonucleotide pair used to generate a synthetic hairpin, recreates an Asp718 site at one of the termini if inserted in Asp718 site:

HP/aspl 5'-GTACACTGACCTAGTGCCGCCCGGGCAAAGCCCGGGCGGCACTAGGTCAG

HP/asp2 5'-GTACCTGACCTAGTGCCGCCCGGGCTTTGCCCGGGCGGCACTAGGTCAGT

Synthetic oligonucleotide pair used to generate a synthetic hairpin, contains the ClaI recognition site to be used for hairpin formation.

HP/clal 5'-GTACATTGACCTAGTGCCGCCCGGGCAAAGCCCGGGCGGCACTAGGTCAATCGAT

HP/cla2 5'-GTACATCGATTGACCTAGTGCCGCCCGGGCTTTGCCCGGGCGGCACTAGGTCAAT

TABLE II

Cell	Passagenumber	IG.Ad.CMV.lacZ	IG.Ad.CMV.TK	IG.Ad.MLPI.TK	dl313	Producer Mean
293		6.0	5.8	24	34	17.5
911		8	14	34	180	59.5
PER.C3	17	8	11	44	40	25.8
PER.C5	15	6	17	36	200	64.7
PER.C6	36	10	22	58	320	102

Yields x 10^{-8} pfu/T175 flask.

Table II.
Yields of different recombinant adenoviruses obtained after inoculation of adenovirus E1 packaging cell lines 293, 911, PER.C3, PER.C5 and PER.C6. The yields are the mean of two different experiments.
IG.Ad.CMV.lacZ and IG.Ad.CMV.TK are described in patent application EP 95 20 2213
The construction of IG.Ad.MLPI.TK is described in this patent application.
Yields of virus per T80 flask were determined by plaque assay on 911 cells, as described [Fallaux, 1996 #1493]

CLAIMS

1. A recombinant nucleic acid molecule based on or derived from an adenovirus having at least a functional encapsidating signal and at least one functional Inverted Terminal Repeat or a functional fragment or derivative thereof and having no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which it is transferred.

2. A recombinant nucleic acid molecule according to claim 1 being in a linear form and comprising an Inverted Terminal Repeat at or near both termini.

3. A recombinant nucleic acid molecule according to claim 1 being in a linear and essentially single stranded form and comprising at the 3' terminus a sequence complementary to an upstream part of the same strand of said nucleic acid molecule, said sequence being capable of base-pairing with said part in a way to be able to function as a start-site for a nucleic acid polymerase.

4. A recombinant nucleic acid molecule according to claim 3, comprising all adenovirus derived genetic information necessary for replication, except for a functional encapsidation signal.

5. A recombinant nucleic acid molecule derived from the nucleic acid molecule according to claim 4 resulting from the action of a nucleic acid polymerase on said nucleic acid molecule according to claim 4.

6. A recombinant nucleic acid molecule according to claim 5 having an Inverted Terminal Repeat at both termini.

7. A recombinant nucleic acid molecule according to anyone of the aforegoing claims comprising a host range mutation.

8. A recombinant nucleic acid molecule according to anyone of the aforegoing claims comprising a mutated E2

region rendering at least one of its products temperature sensitive.

9. A recombinant nucleic acid molecule according to anyone of the aforegoing claims comprising an E2 region under the control of an inducible promoter.

10. A packaging cell for packaging adenovirus derived nucleic acid molecules, which packaging cell has been provided with one or more recombinant nucleic acid molecules which provide said cell with the ability to express adenoviral gene products derived from at least the E1A region.

11. A packaging cell for packaging adenovirus derived nucleic acid molecules, which packaging cell has been provided with one or more recombinant nucleic acid molecules which provide said cell with the ability to express adenoviral gene products derived from at least both the E1A and the E2A region.

12. A packaging cell according to claim 11, wherein the recombinant nucleic acid molecule encoding the E2A region is under control of an inducible promoter.

13. A packaging cell according to claim 11 or 12, wherein the recombinant nucleic acid molecule encoding the E2A region is mutated so that at least one of its products is temperature sensitive.

14. A cell according to anyone of claims 10-13, which does not have the ability to express E1B products.

15. A cell according to claim 14, wherein the genetic information encoding E1B products is not present.

16. A cell according to claim 10, further comprising the region coding for E1B.

17. A cell according to claim 10, further comprising a marker gene.

18. A cell according to claim 17, whereby the marker gene is under control of the E1B responsive promoter.

19. A packaging cell harbouring nucleotides 80-5788 of the human Adenovirus 5 genome.

20. A packaging cell harbouring nucleotides 459-1713 of the human Adenovirus 5 genome.

21. A packaging cell harbouring nucleotides 459-3510 of the human Adenovirus 5 genome.

22. A cell according to anyone of claims 10-13, which does not have the ability to express the 21kD E1B product.

23. A cell according to claim 22, wherein the genetic information encoding the 21kD E1B product is not present.

24. A cell according to anyone of claims 10-23 which is a diploid cell.

25. A cell according to anyone of claims 10-24 which is of non-human origin.

26. A cell according to anyone of claims 10-25 which is of monkey origin.

27. A cell according to claim 19 as deposited under no. 95062101 at the ECACC.

28. A recombinant nucleic acid molecule according to anyone of claims 1-9 being a DNA molecule.

29. A recombinant nucleic acid molecule based on or derived from an adenovirus, having at least a deletion of nucleotides 459-3510 of the E1 region.

30. A recombinant nucleic acid molecule based on or derived from an adenovirus, having a deletion of nucleotides 459-1713 of the E1 region.

31. An adenovirus-like particle comprising a recombinant nucleic acid molecule according to anyone of claims 1-9.

32. A cell comprising a recombinant nucleic acid molecule according to anyone of claims 1-9.

33. A recombinant nucleic acid according to claims 1-3, comprising functional E2A end E2B genes or functional fragments or derivatives thereof under control of an E1A independent promoter.

34. A cell according to claim 26 which comprises a host range mutated E2A region of an adenovirus.

35. A method for intracellular amplification comprising the steps of providing a cell with a linear DNA fragment to be amplified, which fragment is provided with at least

a functional part or derivative of an Inverted Terminal Repeat at one terminus and providing said cell with functional E2 derived products necessary for replication of said fragment and allowing said fragment to be acted upon by a DNA polymerase.

36. A method according to claim 35 whereby the cell is provided with genetic material encoding both E2A and E2B products.

37. A method according to claim 35 or 36 whereby a hairpin-like structure is provided at the terminus of the DNA fragment opposite the Inverted Terminal Repeat.

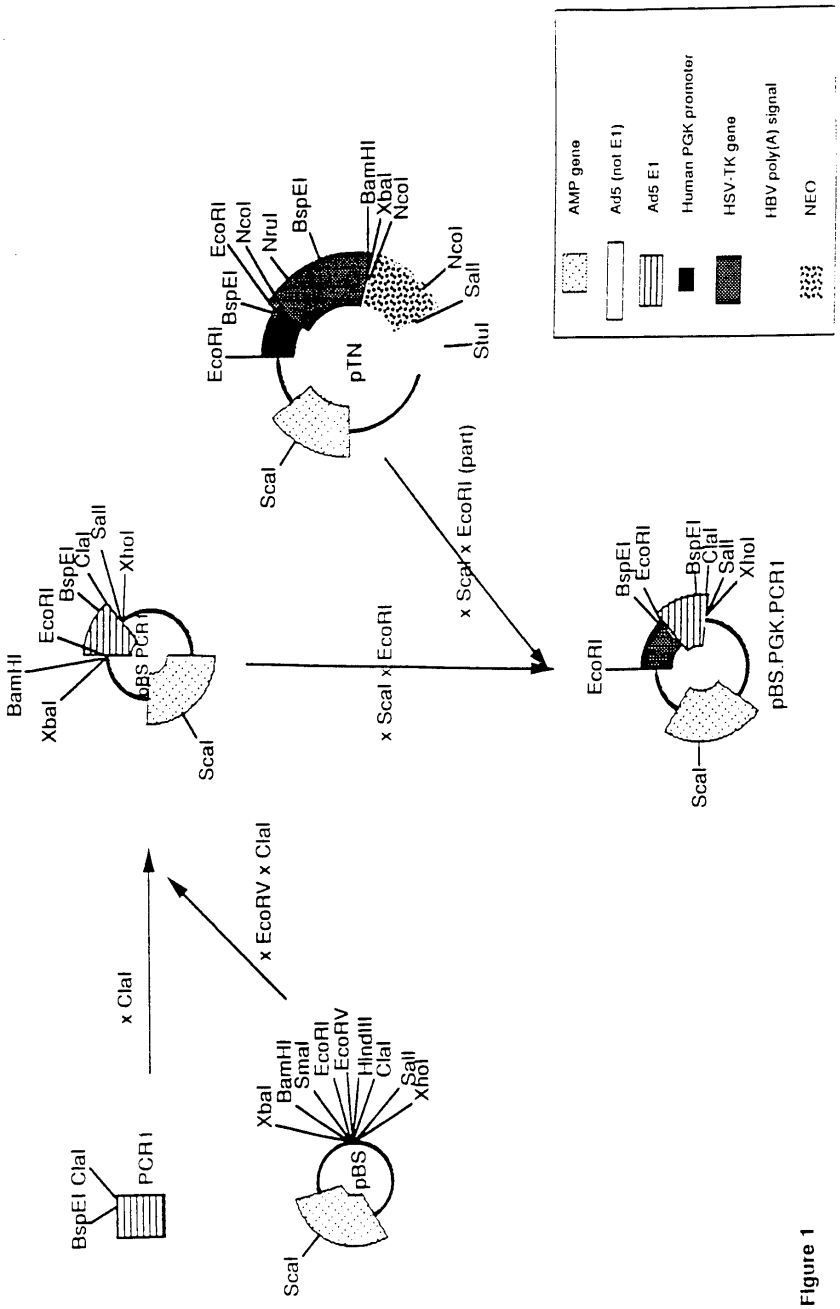


Figure 1

Construction of pBS.PGK.PCR1

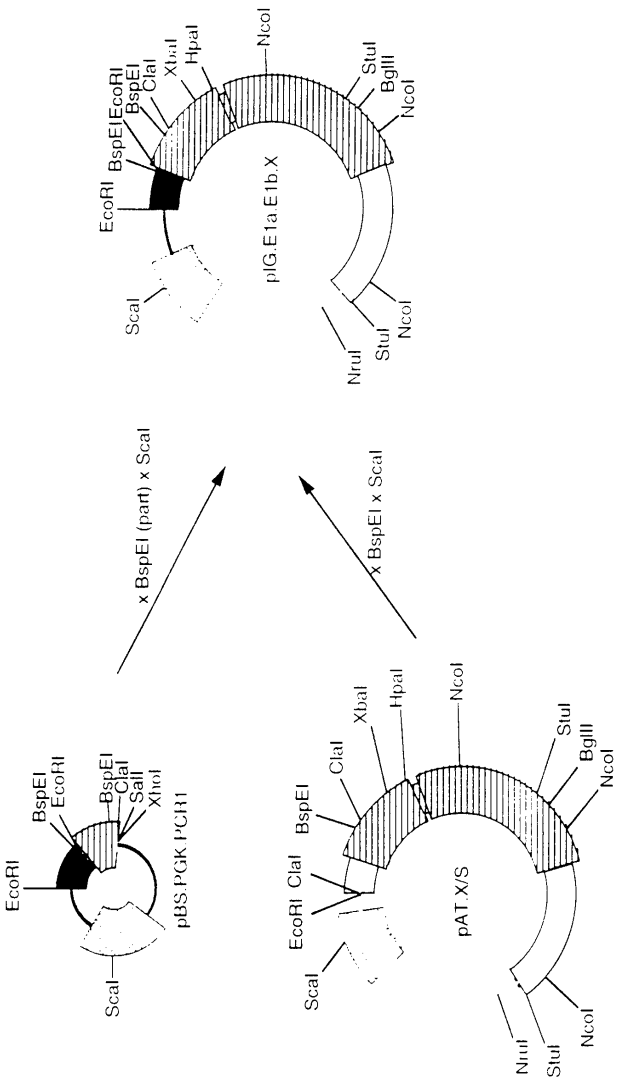


Figure 2

Construction of pIG.E1a.E1b.X

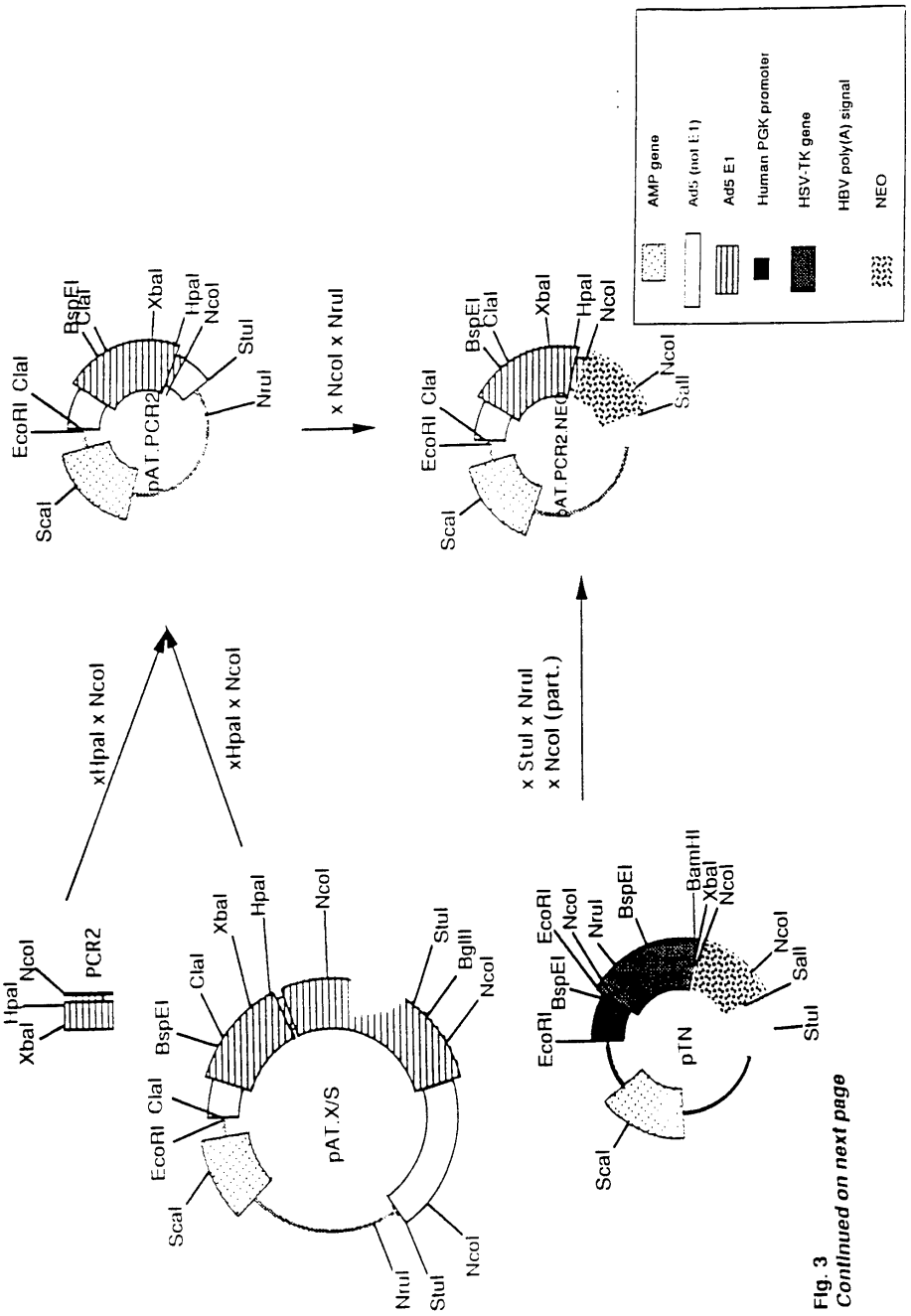


Fig. 3

Continued on next page

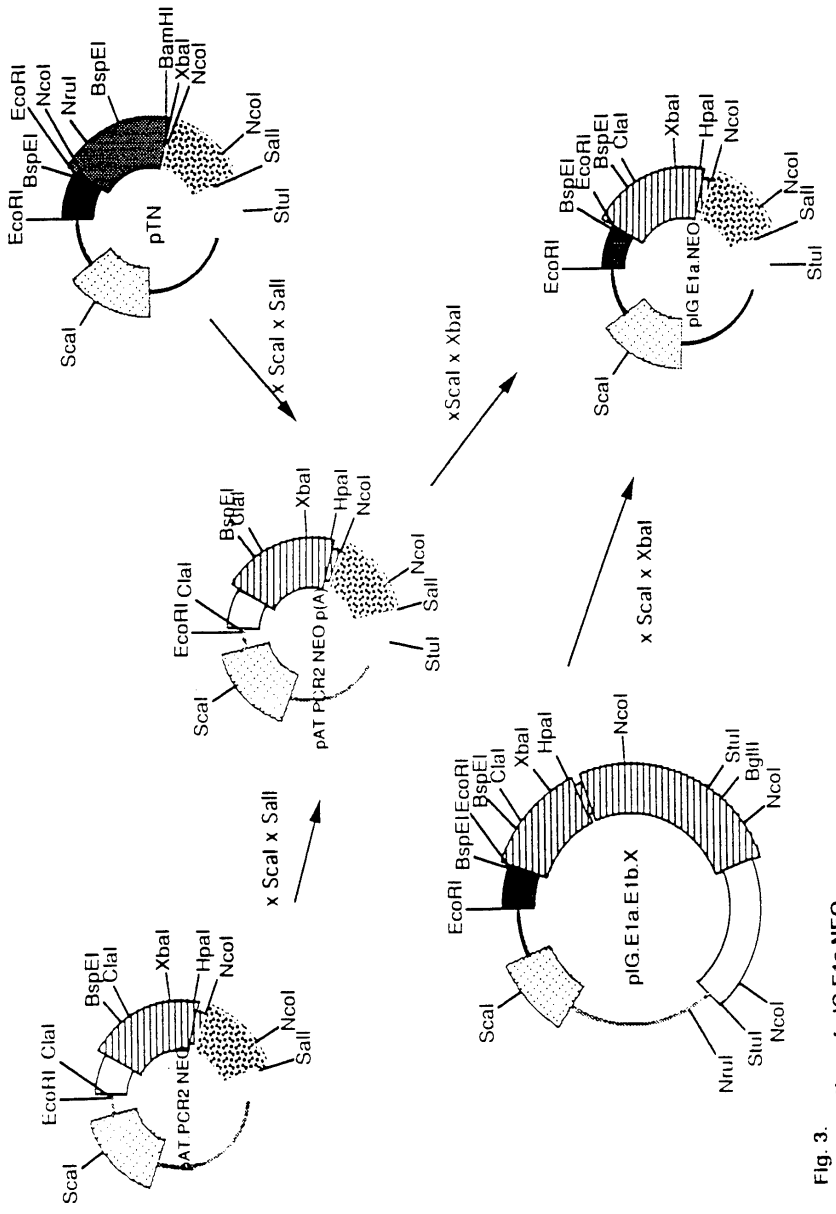


Fig. 3.
Construction of plG.E1a.NEO

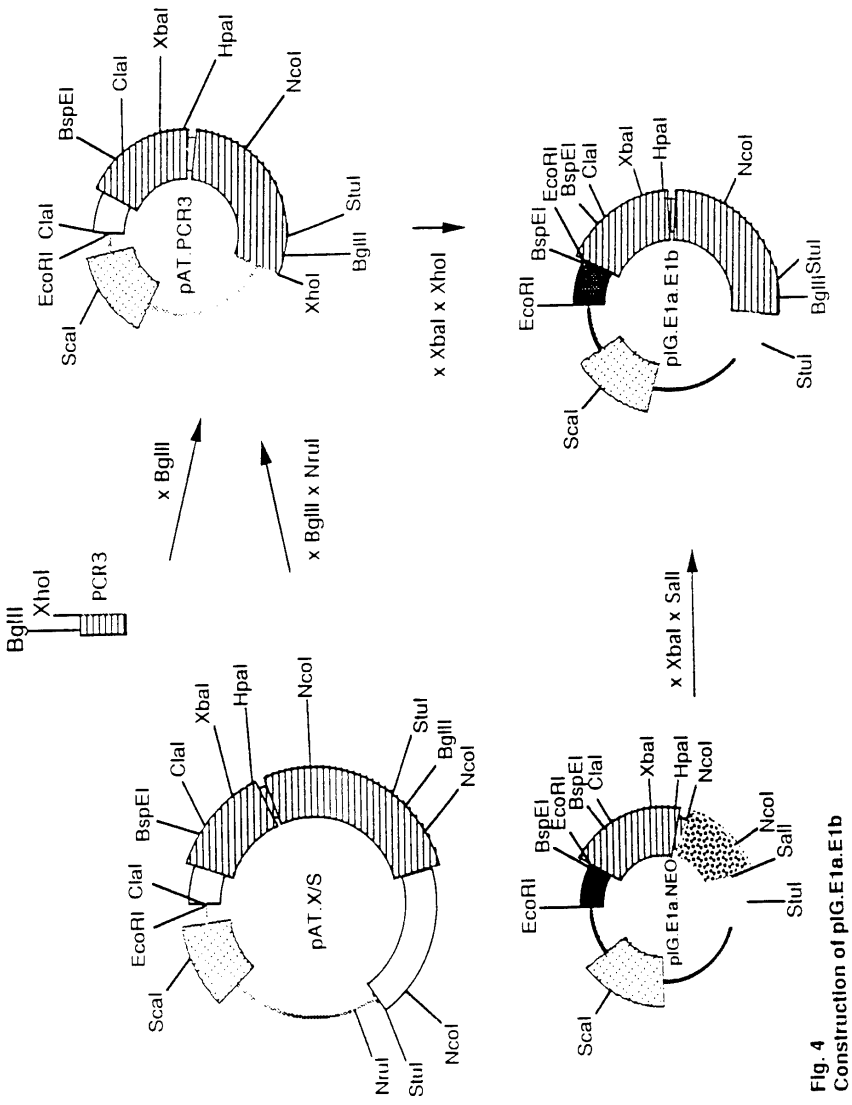


Fig. 4
Construction of pIG.E1a.E1b

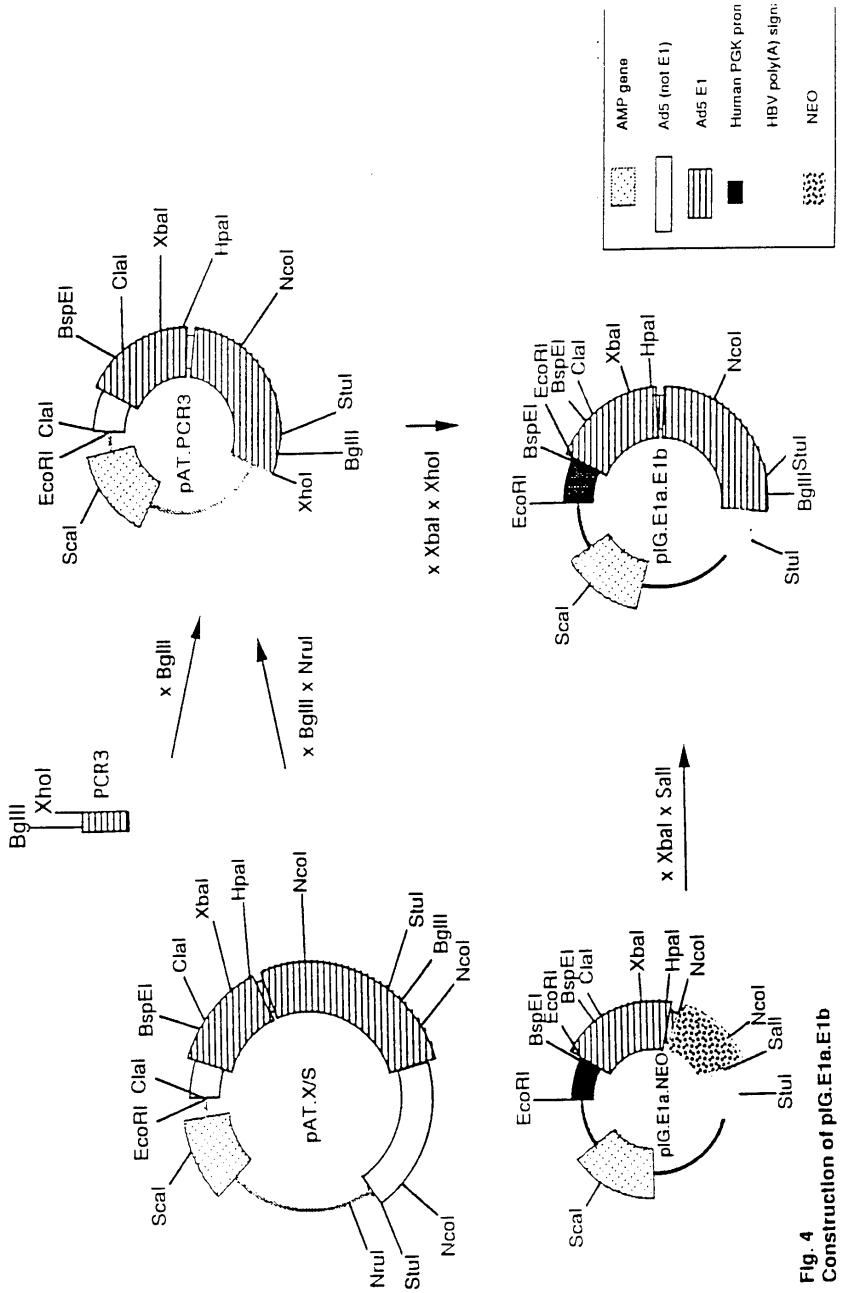


Fig. 4
Construction of pIG.E1a.E1b

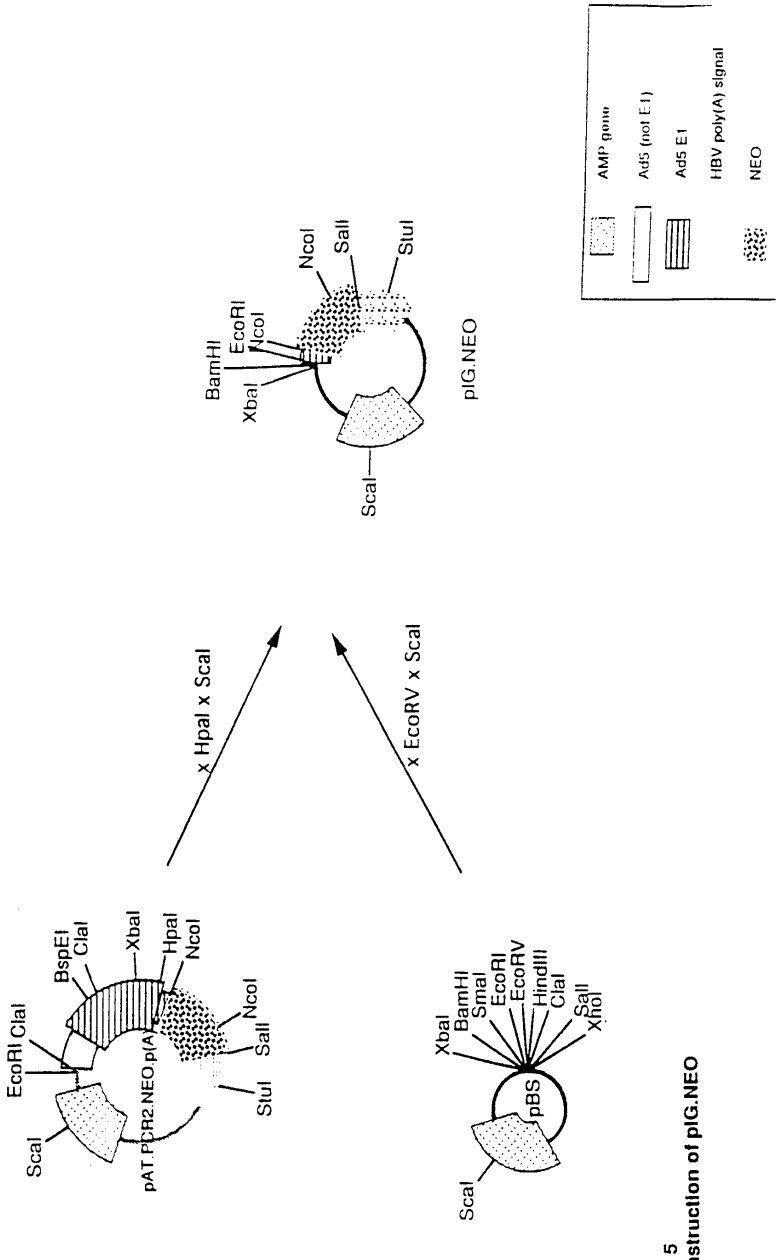


Fig. 5
Construction of pIG.NEO

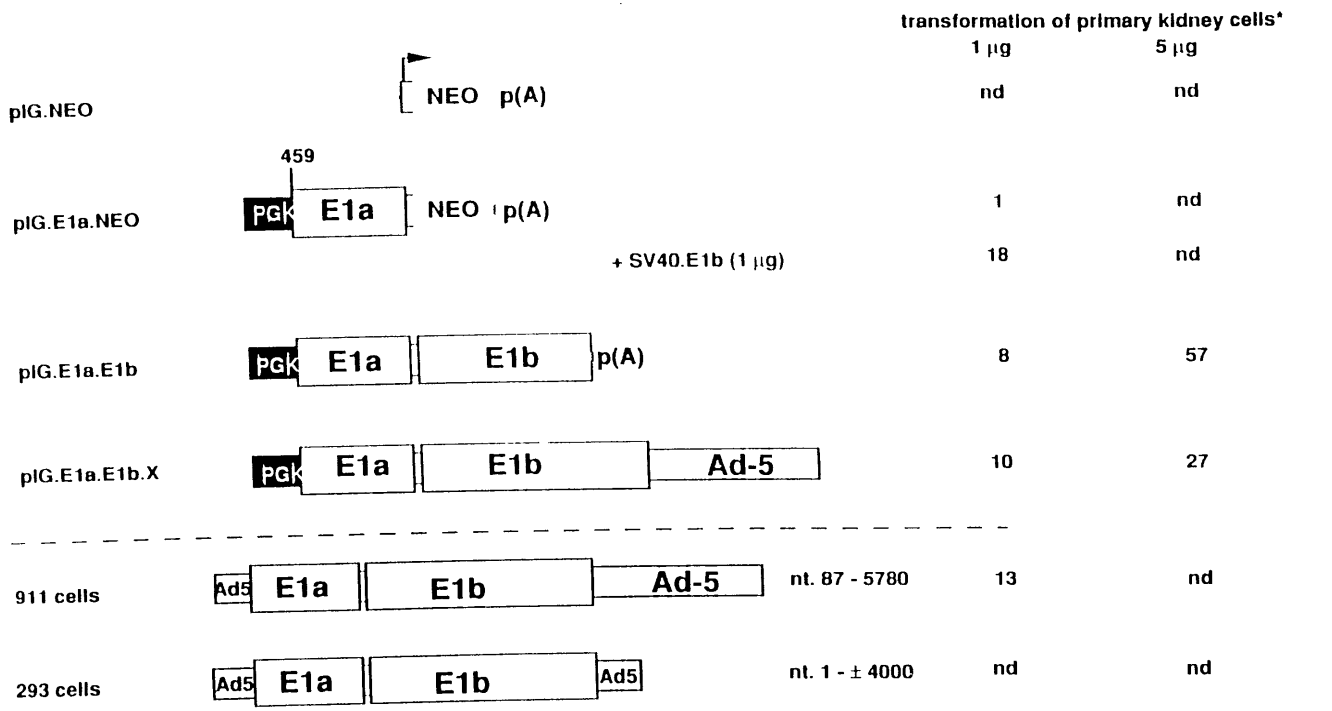


Figure 6
Overview of available adenovirus packaging constructs and assessment of their capacity to transform primary kidney cells

Figure 7

Western blotting analysis of A549 clones transfected with pIG.E1A.NEO and PER clones (HER cells transfected with pIG.E1A.E1B)

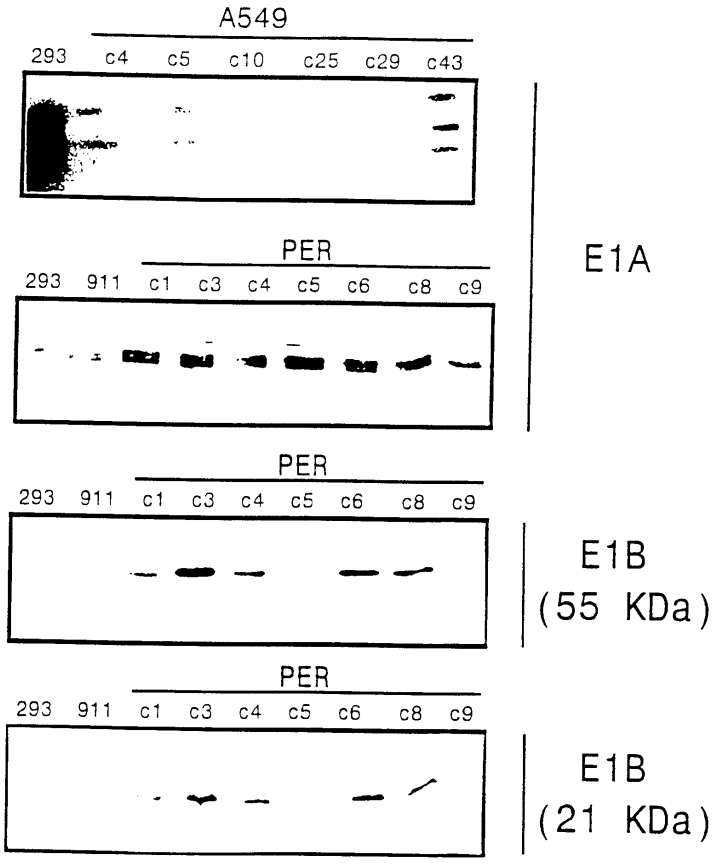


Figure 8

Southern blot analyses of 293, 911 and PER cell lines

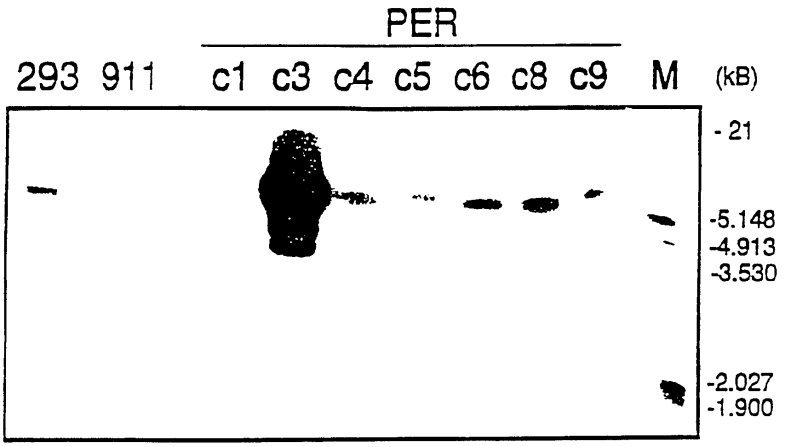
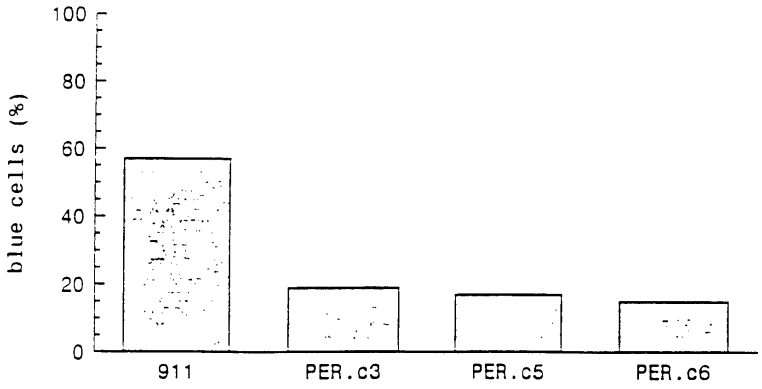


Figure 9

Transfection efficiency of PER.C3, PER.C5, PER.C6 and 911 cells. Cells were cultured in 6-well plates and transfected (n=2) with 5 µg pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours later the cells were stained with X-GAL. The mean percentage of blue cells is shown.



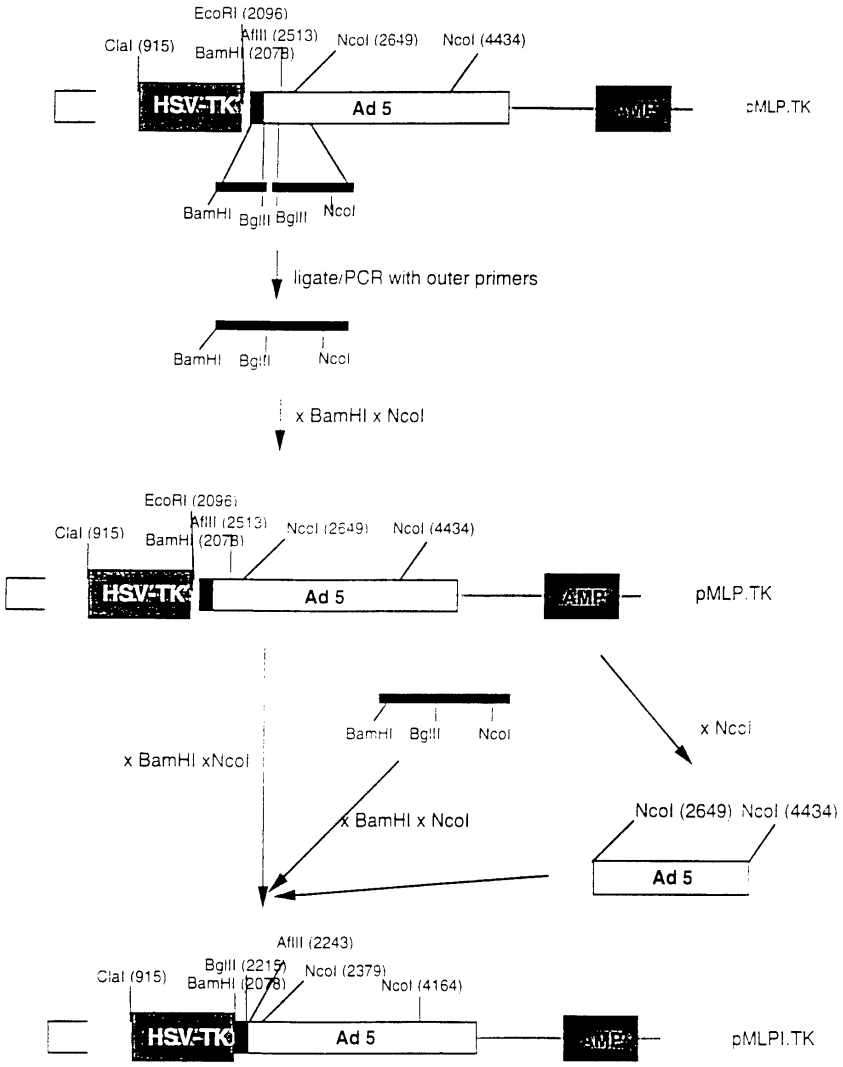


Figure 10.
Construction of pMLPI.TK from pMLP.TK

New recombinant adenoviruses and packaging constructs without sequence overlap

Figure 11a
Packaging system based on primary cells

New recombinant adenoviruses and packaging constructs without sequence overlap

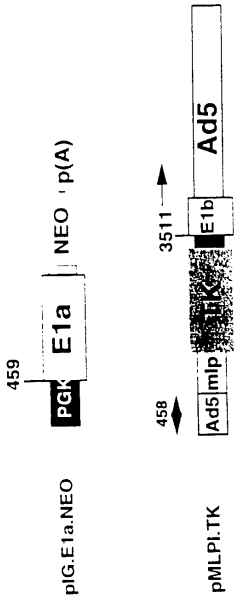


Figure 11.b
Packaging system based on established cell lines: transfection with E1a and selection with G418

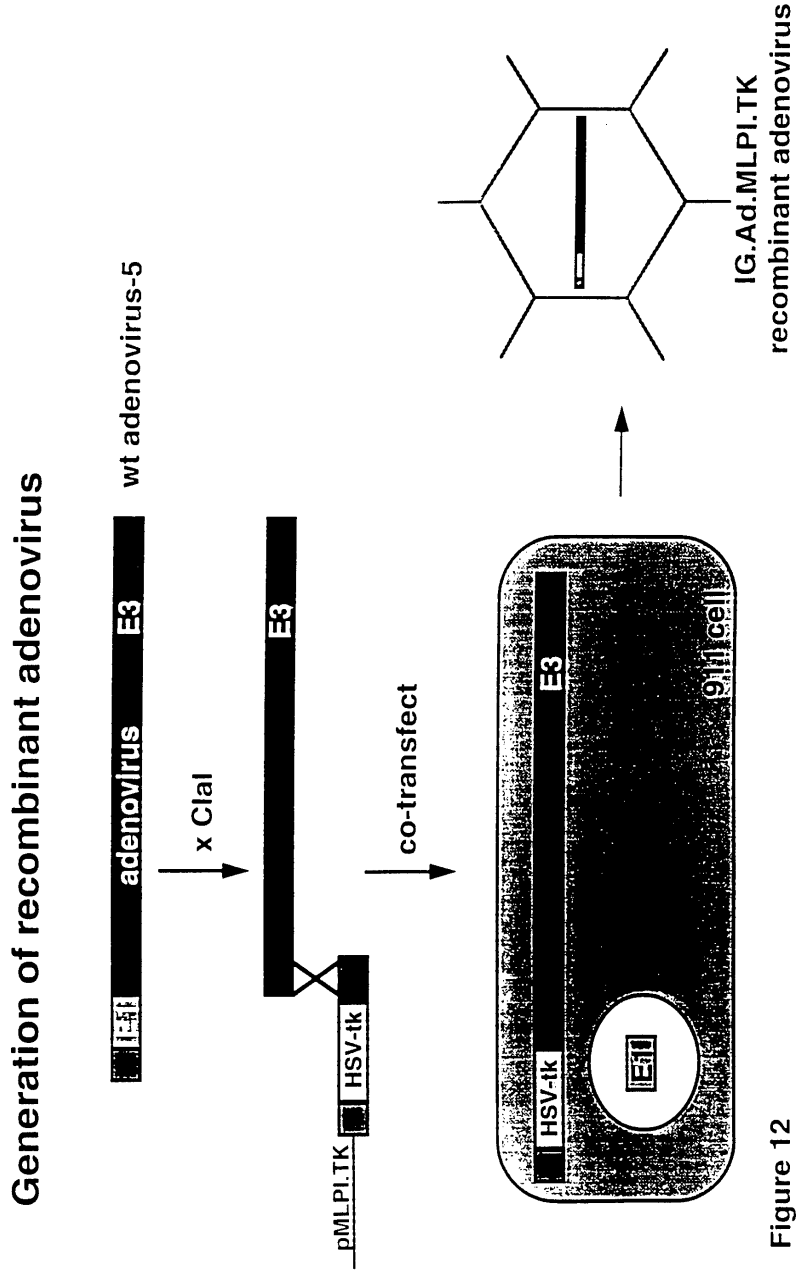

Generation of recombinant adenovirus
wt adenovirus-5
adenovirus
E3
x ClaI
E3
pMLP1.TK
HSV-tk
co-transfect
E3
HSV-tk
E1
911 cell
IG.Ad.MLP1.TK
recombinant adenovirus

Figure 12

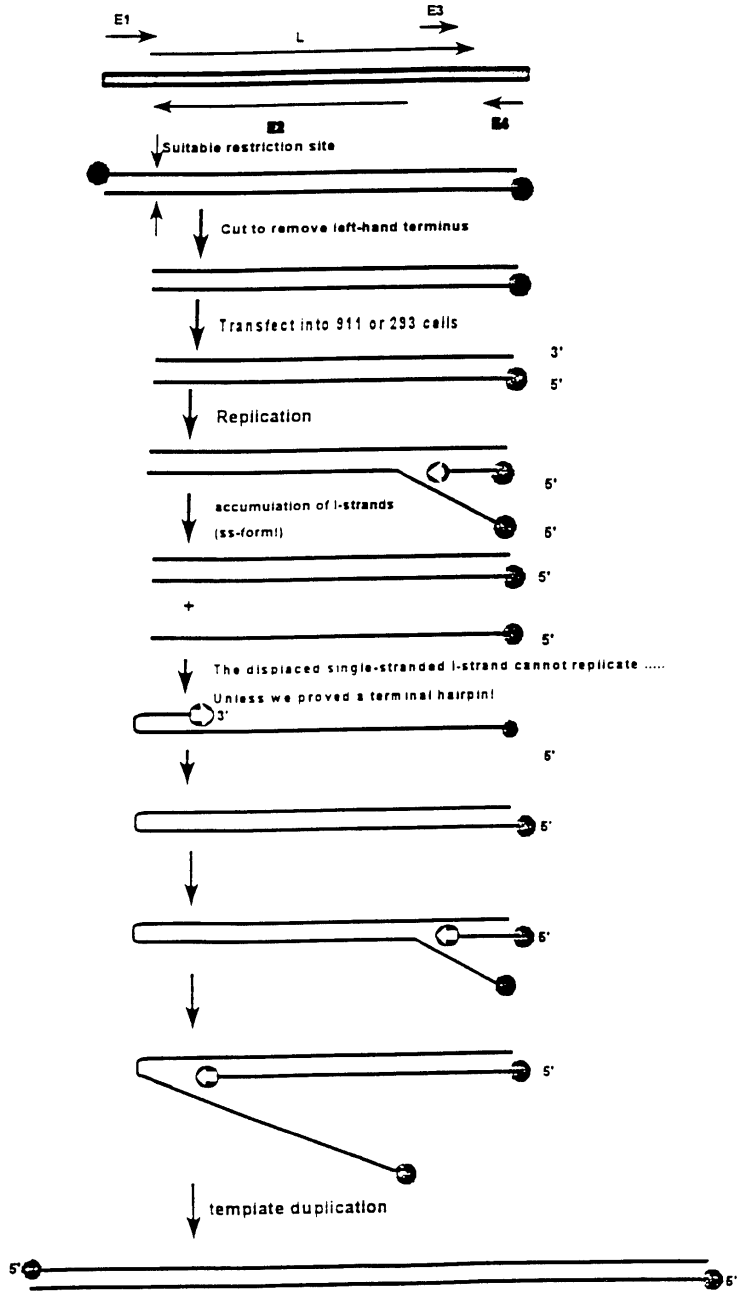

E1
E3
L
E2
E4
Suitable restriction site
Cut to remove left-hand terminus
Transfect into 911 or 293 cells
3'
5'
Replication
5'
accumulation of I-strands
(ss-form!)
5'
5'
+
5'
The displaced single-stranded I-strand cannot replicate .....
Unless we proved a terminal hairpin!
3'
5'
5'
5'
5'
template duplication
5'
5'

Figure 13

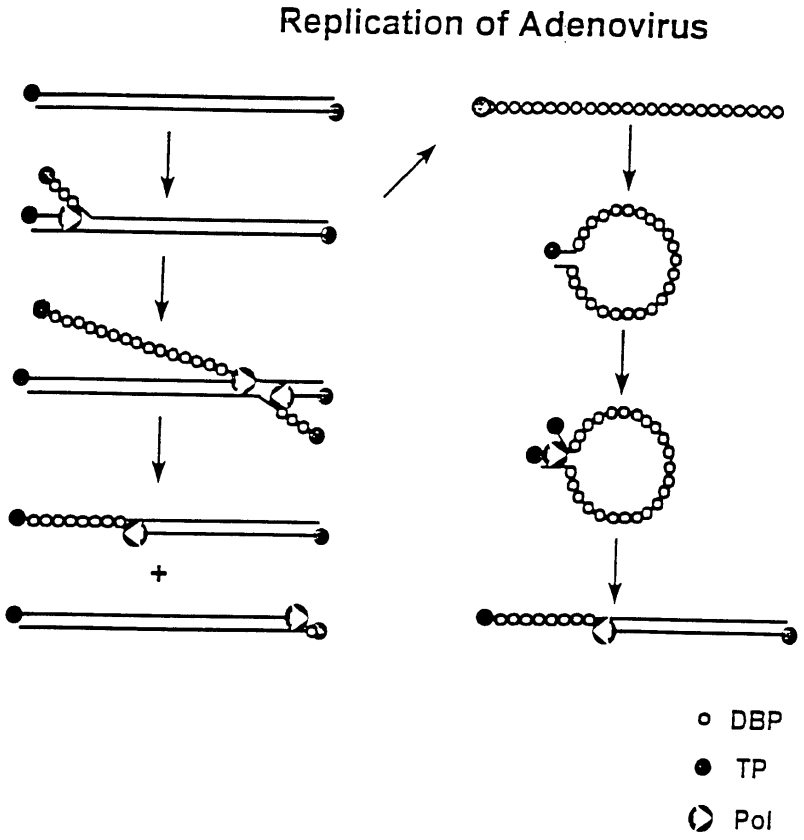


Figure 14

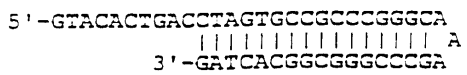


Fig 15. The potential hairpin conformation of a single-stranded DNA molecule that contains the HP/asp sequences used in these studies. Restriction with the restriction endonuclease *Asp*718I of plasmid pICLha$_2$, containing the annealed oligonucleotide pair HP/asp1 en HP/asp2 will yield a linear double-stranded DNA fragment. In cells in which the required adenovirus genes are present, replication can initiate at the terminus that contains the ITR sequence. During the chain elongation, the one of the strands will be displaced. The terminus of the single-stranded displaced- strand molecule can adopt the conformation depicted above. In this conformation the free 3'-terminus can serve as a primer for the cellular and/or adenovirus DNA polymerase, resulting in conversion of the displaced strand in a double-stranded form.

Figure 15

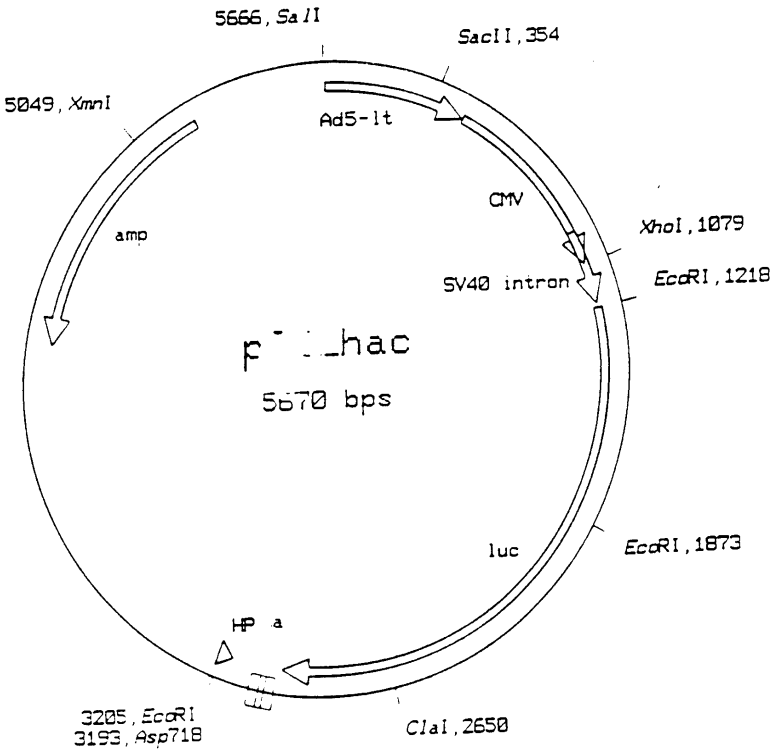

5666, SalI
SacII, 354
5049, XmnI
Ad5-1t
CMV
XhoI, 1079
amp
SV40 intron
EcoRI, 1218
5670 bps
luc
EcoRI, 1873
HP a
3205, EcoRI
3193, Asp718
ClaI, 2650

Figure 16

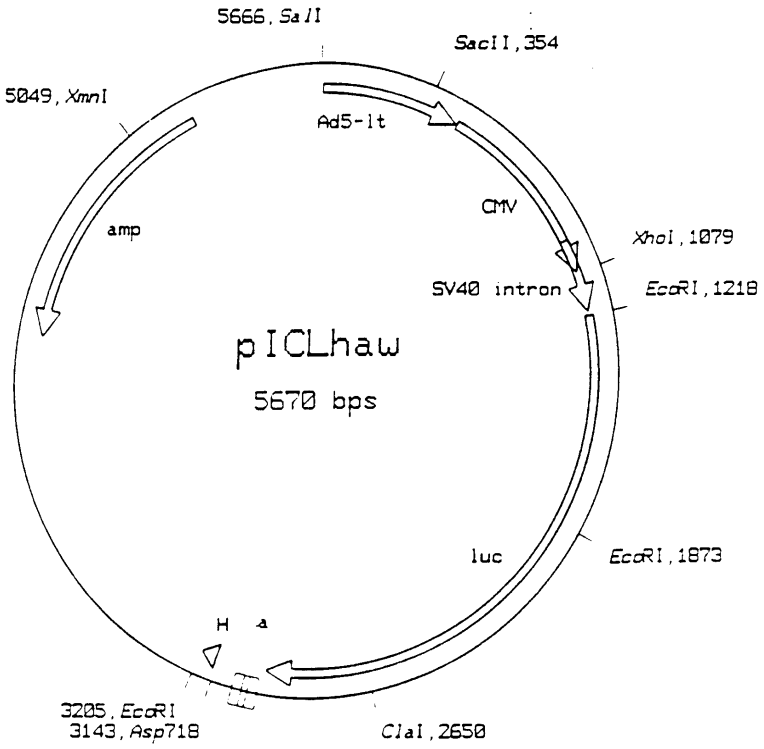

5666, SalI
SacII, 354
5049, XmnI
Ad5-1t
CMV
amp
XhoI, 1079
SV40 intron
EcoRI, 1218
pICLhaw
5670 bps
luc
EcoRI, 1873
H a
3205, EcoRI
3143, Asp718
ClaI, 2650

Figure 17

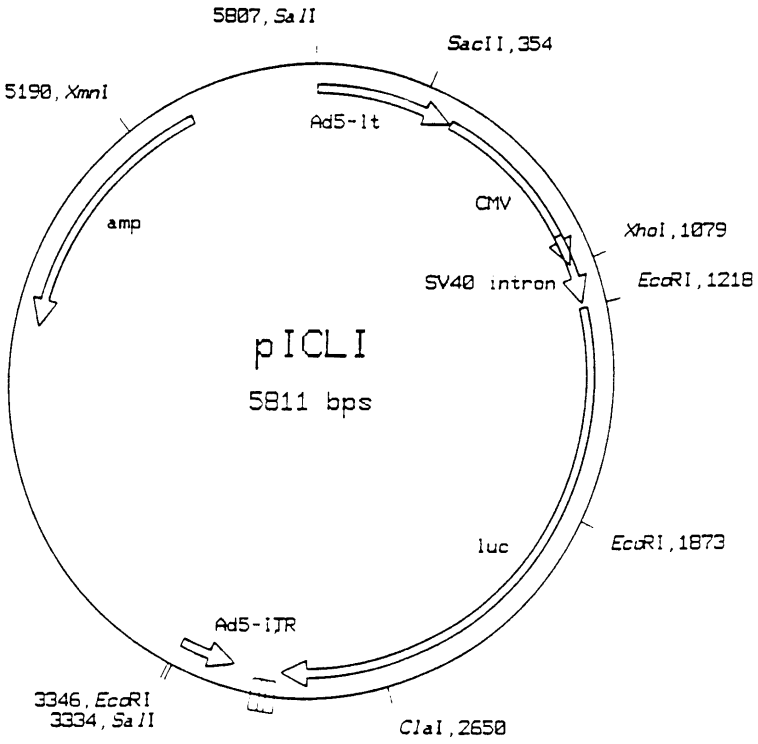

5807, SalI
SacII, 354
5190, XmnI
Ad5-lt
CMV
amp
XhoI, 1079
SV40 intron
EcoRI, 1218
pICLI
5811 bps
luc
EcoRI, 1873
Ad5-iTR
3346, EcoRI
3334, SalI
ClaI, 2650

Figure 18

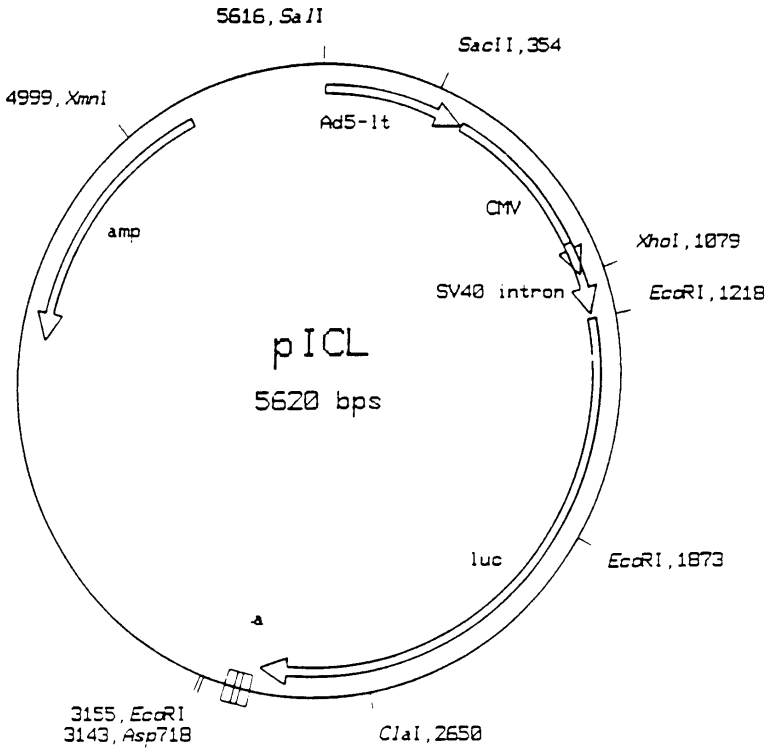

5616, SalI
SacII, 354
4999, XmnI
Ad5-1t
CMV
amp
XhoI, 1079
SV40 intron
EcoRI, 1218
pICL
5620 bps
luc
EcoRI, 1873
3155, EcoRI
3143, Asp718
ClaI, 2650

Figure 19

Plasmid pICL is derived from the following plasmids:

nt.1 - 457 pMLP10 (Levrerno et al.,)
nt.458 - 1218 pCMVß (Clontech, EMBL Bank no. U02451)
nt.1219 - 3016 pMLP.luc (Introgene, unpublished)
nt.3017 - 5620 pBLCAT5 (Stein et al., 1989)

The plasmid has been constructed as follows:

The tet gene of plasmid pMLP10 has been inactivated by deletion of the BamHI-SalI fragment, to generate pMLP10ΔSB. Using primer ser PCR/MLP1 and PCR/MLP3 a 210 bp fragment containing the Ad5-ITR, flanked by a synthetic SalI restriction site was amplified using pMLP10 DNA as the template. The PCR product was digested with the enzymes EcoRI and SgrAI to generate a 196 bp. fragment. Plasmid pMLP10ΔSB was digested with EcoRI and SgrAI to remove the ITR. This fragment was replaced by the EcoRI-SgrAI-treated PCR fragment to generate pMLP/SAL.

Plasmid pCMV-Luc was digested with PvuII to completion and recirculated to remove the SV40-derived poly-adenylation signal and Ad5 sequences with exception of the Ad5 left-terminus. In the resulting plasmid, pCMV-lucΔAd, the Ad5 ITR was replaced by the Sal-site-flanked ITR from plasmid pMLP/SAL by exchanging the xmnI-SacII fragments. The resulting plasmid, pCMV-lucΔAd/SAL, the Ad5 left terminus and the CMV-driven luciferase gene were isolated as an SalI-SmaI fragment and inserted in the SalI and HpaI digested plasmid pBLCAT5, to form plasmid pICL. Plasmid pICL is represented in figure 19

Plasmid pICL contains the following features:

nt. 1-457 Ad5 left terminus (Sequence 1-457 of human adenovirus type 5)
nt. 458-969 Human cytomegalovirus enhancer and immediate early promoter (Bosthart et al., 1985; from plasmid pCMVß)
nt. 970-1204SV40 19S exon and truncated 16/19S intron (from plasmid pCMVß)
nt. 1218-2987 Firefly luciferase gene (from pMLP.luc)
nt. 3018-3131SV40 tandem poly-adenylation signals from late transcript, derived from plasmid pBLCAT5)
nt. 3132-5620 pUC12 backbone (derived from plasmid pBLCAT5)
nt. 4337-5191ß-lactamase gene (Amp-resistence gene, reverse orientation)

NAME: pICL 5620 BPS DNA CIRCULAR UPDATED 5/01/95
DESCRIPTION: 1 x Ad5-ITR, CMV-luciferase, minimal vector
SEQUENCE: sequence based on the on available information;
Constructions verified by restriction enzyme digests;
Sequence of regions derived from amplified DNA verified by sequence analyses

* * * S E Q U E N C E * * *

```
   1  CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT
  61  TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT
 121  GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGACGGATG TGGCAAAAGT GACGTTTTTG
 181  GTGTGCGCCG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG
 241  TAAATTTGGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG AATAAGAGGA
 301  AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG
 361  GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC
 421  CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGGGGCTG CAGGTCGTTA CATAACTTAC
 481  GGTAAATGGC CCGCCTGGCT GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC
 541  GTATGTTCCC ATAGTAACGC CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGAGTATTT
 601  ACGGTAAACT GCCCACTTGG CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT
 661  TGACGTCAAT GACGGTAAAT GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA
 721  CTTTCCTACT TGGCAGTACA TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT
 781  TTGGCAGTAC ATCAATGGGC GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA
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 841  CCCCATTGAC GTCAATGGGA GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG
 901  TCGTAACAAC TCCGCCCCAT TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA
 961  TATAAGCAGA GCTCGTTTAG TGAACCGTCA GATCGCCTGG AGACGCCATC CACGCTGTTT
1021  TGACCTCCAT AGAAGACACC GGGACCGATC CAGCCTCCGG ACTCTAGAGG ATCCGGTACT
1081  CGAGGAACTG AAAAACCAGA AAGTTAACTG GTAAGTTTAG TCTTTTTGTC TTTTATTTCA
1141  GGTCCCGGAT CCGGTGGTGG TGCAAATCAA AGAACTGCTC CTCAGTGGAT GTTGCCTTTA
1201  CTTCTAGTAT CAAGCTTGAA TTCCTTTGTG TTACATTCTT GAATGTCGCT CGCAGTGACA
1261  TTAGCATTCC GGTACTGTTG GTAAAATGGA AGACGCCAAA AACATAAAGA AAGGCCCGGC
1321  GCCATTCTAT CCTCTAGAGG ATGGAACCGC TGGAGAGCAA CTGCATAAGG CTATGAAGAG
1381  ATACGCCCTG GTTCCTGGAA CAATTGCTTT TACAGATGCA CATATCGAGG TGAACATCAC
1441  GTACGCGGAA TACTTCGAAA TGTCCGTTCG GTTGGCAGAA GCTATGAAAC GATATGGGCT
1501  GAATACAAAT CACAGAATCG TCGTATGCAG TGAAAACTCT CTTCAATTCT TTATGCCGGT
1561  GTTGGGCGCG TTATTTATCG GAGTTGCAGT TGCGCCCGCG AACGACATTT ATAATGAACG
1621  TGAATTGCTC AACAGTATGA ACATTTCGCA GCCTACCGTA GTGTTTGTTT CCAAAAAGGG
1681  GTTGCAAAAA ATTTTGAACG TGCAAAAAAA ATTACCAATA ATCCAGAAAA TTATTATCAT
1741  GGATTCTAAA ACGGATTACC AGGGATTTCA GTCGATGTAC ACGTTCGTCA CATCTCATCT
1801  ACCTCCCGGT TTTAATGAAT ACGATTTTGT ACCAGAGTCC TTTGATCGTG ACAAAACAAT
1861  TGCACTGATA ATGAATTCCT CTGGATCTAC TGGGTTACCT AAGGGTGTGG CCCTTCCGCA
1921  TAGAACTGCC TGCGTCAGAT TCTCGCATGC CAGAGATCCT ATTTTTGGCA ATCAAATCAT
1981  TCCGGATACT GCGATTTTAA GTGTTGTTCC ATTCCATCAC GGTTTTGGAA TGTTTACTAC
2041  ACTCGGATAT TTGATATGTG GATTTCGAGT CGTCTTAATG TATAGATTTG AAGAAGAGCT
2101  GTTTTTACGA TCCCTTCAGG ATTACAAAAT TCAAAGTGCG TTGCTAGTAC CAACCCTATT
2161  TTCATTCTTC GCCAAAAGCA CTCTGATTGA CAAATACGAT TTATCTAATT TACACGAAAT
2221  TGCTTCTGGG GGCGCACCTC TTTCGAAAGA AGTCGGGGAA GCGGTTGCAA AACGCTTCCA
2281  TCTTCCAGGG ATACGACAAG GATATGGGCT CACTGAGACT ACATCAGCTA TTCTGATTAC
2341  ACCCGAGGGG GATGATAAAC CGGGCGCGGT CGGTAAAGTT GTTCCATTTT TTGAAGCGAA
2401  GGTTGTGGAT CTGGATACCG GGAAAACGCT GGGCGTTAAT CAGAGAGGCG AATTATGTGT
2461  CAGAGGACCT ATGATTATGT CCGGTTATGT AAACAATCCG GAAGCGACCA ACGCCTTGAT
2521  TGACAAGGAT GGATGGCTAC ATTCTGGAGA CATAGCTTAC TGGGACGAAG ACGAACACTT
2581  CTTCATAGTT GACCGCTTGA AGTCTTTAAT TAAATACAAA GGATATCAGG TGGCCCCCGC
2641  TGAATTGGAA TCGATATTGT TACAACACCC CAACATCTTC GACGCGGGCG TGGCAGGTCT
2701  TCCCGACGAT GACGCCGGTG AACTTCCCGC CGCCGTTGTT GTTTTGGAGC ACGGAAAGAC
2761  GATGACGGAA AAAGAGATCG TGGATTACGT CGCCAGTCAA GTAACAACCG CGAAAAAGTT
2821  GCGCGGAGGA GTTGTGTTTG TGGACGAAGT ACCGAAAGGT CTTACCGGAA AACTCGACGC
2881  AAGAAAAATC AGAGAGATCC TCATAAAGGC CAAGAAGGGC GGAAAGTCCA AATTGTAAAA
2941  TGTAACTGTA TTCAGCGATG ACGAAATTCT TAGCTATTGT AATGGGGGAT CCCCAACTTG
3001  TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA
3061  GCATTTTTTT CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT
3121  GTCTGGATCG GATCGATCCC CGGGTACCGA GCTCGAATTC GTAATCATGG TCATAGCTGT
3181  TTCCTGTGTG AAATTGTTAT CCGCTCACAA TTCCACACAA CATACGAGCC GGAAGCATAA
3241  AGTGTAAAGC CTGGGGTGCC TAATGAGTGA GCTAACTCAC ATTAATTGCG TTGCGCTCAC
3301  TGCCCGCTTT CCAGTCGGGA AACCTGTCGT GCCAGCTGCA TTAATGAATC GGCCAACGCG
3361  CGGGGAGAGG CGGTTTGCGT ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC
3421  GCTCGGTCGT TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT
3481  CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA
3541  GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG GCTCCGCCCC CCTGACGAGC
3601  ATCACAAAAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC GACAGGACTA TAAAGATACC
3661  AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG
3721  GATACCTGTC CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA
3781  GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG
3841  TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC
3901  ACGACTTATC GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG AGGTATGTAG
3961  GCGGTGCTAC AGAGTTCTTG AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT
4021  TTGGTATCTG CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT
4081  CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG CAGATTACGC
4141  GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT
4201  GGAACGAAAA CTCACGTTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG ATCTTCACCT
4261  AGATCCTTTT AAATTAAAAA TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT
4321  GGTCTGACAG TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC
4381  GTTCATCCAT AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC
4441  CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT
4501  CAGCAATAAA CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA ACTTTATCCG
4561  CCTCCATCCA GTCTATTAAT TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA
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4621  GTTTGCGCAA CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA
4681  TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT
4741  GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG
4801  TGTTATCACT CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG CCATCCGTAA
4861  GATGCTTTTC TGTGACTGGT GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC
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5041  TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA
5101  CTTTCACCAG CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA AAAAAGGGAA
5161  TAAGGGCGAC ACGGAAATGT TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA
5221  TTTATCAGGG TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC
5281  AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA
5341  TTATCATGAC ATTAACCTAT AAAAATAGGC GTATCACGAG GCCTATGCGG TGTGAAATAC
5401  CGCACAGATG CGTAAGGAGA AAATACCGCA TCAGGCGCCA TTCGCCATTC AGGCTGCGCA
5461  ACTGTTGGGA AGGGCGATCG GTGCGGGCCT CTTCGCTATT ACGCCAGCTG GCGAAAGGGG
5521  GATGTGCTGC AAGGCGATTA AGTTGGGTAA CGCCAGGGTT TTCCCAGTCA CGACGTTGTA
5581  AAACGACGGC CAGTGCCAAG CTTGCATGCC TGCAGGTCGA
```

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/NL 96/00244

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 28152 (TRANSGENE S.A.) 8 December 1994 see page 9, last paragraph ---	1-6, 28-33
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, WASHINGTON US, pages 6196-6200, XP002012303 ENGELHARDT ET AL.: "Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver" cited in the application see page 6200, right-hand column ---	8,35-37
X	WO,A,95 02697 (RHONE-POULENC RORER) 26 January 1995 see example 4 ---	9-12, 14-24
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1996	06.09.96

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/NL 96/00244

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 34671 (GENVEC INC.) 21 December 1995 see figure 1	1-6, 9-12, 14-24, 28-33, 35-37
P,X	WO,A,95 27071 (BOARDS OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 12 October 1995 see page 7, line 9 - page 16; claims 1-27; examples 3-7	1-3, 9-12, 16-24, 28-33, 35-37
A	JOURNAL OF VIROLOGY, vol. 55, pages 206-212, XP002012304 BROUGH ET AL.: "Restricted changes in the adenovirus DNA-binding protein that lead to extended host range or temperature-sensitive phenotypes" cited in the application see the whole document	7,25,26, 34

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/NL 96/00244

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9428152	08-12-94	FR-A-	2705686	02-12-94
		AU-B-	6850394	20-12-94
		CA-A-	2141212	08-12-94
		EP-A-	0652968	17-05-95
		JP-T-	7509616	26-10-95
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		CZ-A-	9500639	15-11-95
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		NZ-A-	269156	26-03-96
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E.2 WO 94/28152

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(81) Etats désignés: AU, CA, JP, US, brevet européen (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Publiée
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(54) Title: DEFECTIVE ADENOVIRUSES AND CORRESPONDING COMPLEMENTATION LINES

(54) Titre: ADENOVIRUS DEFECTIFS ET LIGNEES DE COMPLEMENTATION CORRESPONDANTES

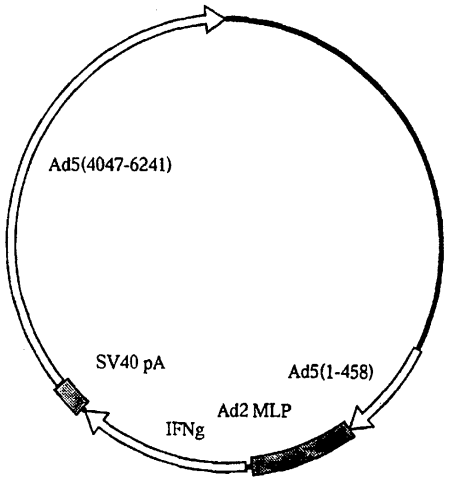
(57) Abstract

Novel defective adenoviruses for the transfer and expression of an exogenous nucleotide sequence in a host cell or organism. The invention also relates to novel complementation lines and to the process for the preparation of these novel defective adenoviruses and their use in therapy and to a pharmaceutical composition containing same.

(57) Abrégé

La présente invention a pour objet de nouveaux adénovirus défectifs pour le transfert et l'expression d'une séquence nucléotidique exogène dans une cellule ou un organisme hôte. L'invention est également relative à de nouvelles lignées de complémentation et le procédé de préparation de ces nouveaux adénovirus défectifs ainsi que leur usage thérapeutique et une composition pharmaceutique les contenant.

pTG6303



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Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

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FR	France	**MN**	Mongolie	**VN**	Viet Nam
GA	Gabon				

ADENOVIRUS DEFECTIFS ET LIGNEES DE COMPLEMENTATION CORRESPONDANTES

L'invention a pour objet de nouveaux vecteurs adénoviraux défectifs permettant le transfert et l'expression de gènes d'intérêt dans une cellule ou un organisme eucaryote hôte ainsi que de nouvelles lignées de complémentation complémentant *en trans* les fonctions virales essentielles qui ont été délétées du génome de ces adénovirus recombinants. L'invention présente un intérêt tout particulier pour des perspectives de thérapie génique, notamment chez l'homme.

Les adénovirus sont des virus à ADN qui présentent un large spectre d'hôte. Ils ont été mis en évidence dans de nombreuses espèces animales et de nombreux types cellulaires. Il existe plusieurs sérotypes qui diffèrent notamment au niveau de la séquence de leurs génomes. La plupart des adénovirus humains sont peu pathogènes et ne produisent généralement que des symptômes bénins.

L'adénovirus pénètre dans la cellule hôte permissive par l'intermédiaire d'un récepteur spécifique, puis il est internalisé et passe dans des endosomes. Leur acidification contribue à un changement de conformation du virus et à sa sortie dans le cytoplasme. Puis, l'ADN viral associé à certaines protéines virales nécessaires aux premières étapes du cycle réplicatif, pénètre dans le noyau des cellules infectées où sa transcription est initiée par des enzymes cellulaires. La réplication de l'ADN adénoviral a lieu dans le noyau des cellules infectées et ne nécessite pas la réplication cellulaire. L'assemblage des nouveaux virions prend également place dans le noyau. Dans un premier temps, les protéines virales s'assemblent de manière à former des capsides vides de structure

icosaedrique, dans lesquelles l'ADN adénoviral est ensuite encapsidé. Les particules virales ou virions sont libérés des cellules infectées et sont susceptibles d'infecter d'autres cellules permissives.

Le cycle infectieux de l'adénovirus s'effectue en 2 étapes :

- la phase précoce qui précède l'initiation de la réplication du génome adénoviral et qui permet la production des protéines régulatrices intervenant au niveau de la réplication et de la transcription de l'ADN viral, et

- la phase tardive qui conduit à la synthèse des protéines structurales.

D'une manière générale, le génome adénoviral est constitué d'une molécule d'ADN linéaire, bicaténaire et d'environ 36kb de long qui contient les séquences codant pour plus de 30 protéines. A chacune de ses extrémités, est présente une courte séquence de 100 à 150 nucléotides selon les sérotypes, inversée et désignée ITR (Inverted Terminal Repeat). Les ITRs sont impliqués dans la réplication du génome adénoviral. La région d'encapsidation, d'environ 300 nucléotides, est située à l'extrémité 5' du génome juste après l'ITR 5'.

Les gènes précoces sont répartis en 4 régions qui sont dispersées dans le génome adénoviral, désignées E1 à E4 (E pour "Early" signifiant précoce en anglais). Les régions précoces comprennent au moins six unités transcriptionnelles qui possèdent leurs propres promoteurs. L'expression des gènes précoces est elle même régulée, certains gènes étant exprimés avant d'autres. Trois régions, respectivement E1, E2 et E4, sont essentielles à la replication virale. Ainsi, si un adénovirus est défectif pour l'une de ces fonctions, c'est à dire s'il ne peut pas produire au moins une protéine codée par l'une de ces régions, celle-ci devra lui être fournie *en trans*.

La région précoce E1 est située à l'extrémité 5' du génome adénoviral et contient 2 unités de transcription virales, respectivement E1A et E1B. Cette région code pour des protéines qui interviennent très précocement dans le cycle viral et sont essentielles à l'expression de presque tous les autres gènes de l'adénovirus. En particulier, l'unité de transcription E1A code pour une protéine trans-activatrice de la transcription des autres gènes viraux, qui induit la transcription à partir des promoteurs des régions E1B, E2A, E2B et E4.

Les produits de la région E2, laquelle comprend également deux unités de transcription E2A et E2B, sont directement impliqués dans la réplication de l'ADN viral. Cette région gouverne notamment la synthèse d'une protéine de 72kDa, qui présente une forte affinité pour l'ADN simple brin et d'une ADN polymérase.

La région E3 n'est pas essentielle à la réplication du virus. Elle code pour au moins six protéines qui seraient responsables de l'inhibition de la réponse immune de l'hôte vis à vis d'une infection par adénovirus. En particulier, la glycoprotéine gp19kDa empêcherait la réponse CTL, responsable de la cytolyse des cellules infectées par les cellules T cytotoxiques de l'hôte.

La région E4 est située à l'extrémité 3' du génome adénoviral. Elle code pour de nombreux polypeptides qui sont impliqués dans l'expression des gènes tardifs, la stabilité des messagers (ARNm) tardifs, le passage de la phase précoce à la phase tardive ainsi que l'inhibition de la synthèse protéique cellulaire.

Une fois la réplication de l'ADN viral initiée, la transcription des gènes tardifs débute. Ceux-ci occupent la majorité du génome adénoviral et recouvrent en partie les unités de transcription des gènes précoces. Mais ils sont transcrits à partir de promoteurs différents et selon un mode d'épissage alternatif, de sorte que les mêmes séquences sont utilisées à des fins différentes. La plupart des gènes tardifs sont transcrits à partir du promoteur majeur tardif MLP (Major Late Promoter). Ce promoteur permet la synthèse d'un long transcrit primaire qui est ensuite maturé en une vingtaine d'ARN messagers (ARNm) à partir desquels sont produites les protéines capsidaires du virion. Le gène codant pour la protéine structurale IX composant la capside est situé à l'extrémité 5' du génome adénoviral et recouvre la région E1B à son extrémité 3'. L'unité transcriptionnelle de la protéine IX utilise le même signal de terminaison de la transcription que l'unité transcriptionnelle E1B.

Un certain nombre d'adénovirus sont maintenant bien caractérisés génétiquement et biochimiquement. Tel est le cas de l'adénovirus humain de type 5 (Ad5) dont la séquence est divulguée dans la banque de donnée Genebank sous la référence M73260. Les différents gènes ont pu être localisés précisément sur le génome adénoviral qui comprend de 5' vers 3' l'ITR 5' de 103 bp suivi de la région d'encapsidation (Hearing et al., 1987, J. Virol., *61*, 2555-2558) d'environ 300 pb, puis des régions précoces et tardives dont l'emplacement est schématiquement représenté dans la Figure 1, et enfin de l'ITR 3'.

Il ressort de ce qui précède que les adénovirus possèdent des caractéristiques intéressantes qui font d'eux des vecteurs de choix pour le transfert de gènes d'intérêt. De nombreux adénovirus recombinants sont décrits dans la littérature (Rosenfeld et al., 1991, Science, *252*, 431-434 ; Rosenfeld et al., 1992, Cell, *68*, 143-155). D'une manière générale, ils dérivent de l'Ad5 et sont défectifs pour la fonction E1, afin d'éviter leur dissémination dans l'environnement et l'organisme hôte. En outre, la région E3 non-essentielle peut également être délétée. Les séquences exogènes sont intégrées à la place de la région E1 ou E3.

Ainsi, ces adénovirus défectifs ne peuvent être propagés que dans une lignée cellulaire complémentant *en trans* la fonction E1 essentielle à la réplication virale. A l'heure actuelle, la seule lignée de complémentation utilisable est la lignée de rein embryonnaire 293 (Graham et al., 1977, J. Gen. Virol., *36*, 59-72), qui résulte de l'intégration dans ses chromosomes, d'un fragment du génome de l'Ad5 comprenant notamment l'extrémité 5' du génome viral ; de sorte que la lignée 293 complémente les adénovirus défectifs pour la fonction E1. Les cellules 293 contiennent des séquences qui se trouvent aussi dans l'adénovirus recombinant défectif, comme l'ITR 5', la région d'encapsidation et la partie en 3' de la région E1B comportant des séquences codant pour les protéines précoces .

La faisabilité du transfert de gènes en utilisant des adénovirus est maintenant établie. Mais, la question de leur innocuité reste posée. En effet, ils sont capables de transformer certaines lignées cellulaires en culture, ce qui reflète le pouvoir potentiellement oncogène de certains des produits d'expression du génome adénoviral, essentiellement de la région E1 et probablement E4, au moins pour certains sérotypes. De plus, la probabilité de recombinaison génétique entre un adénovirus défectif de l'art antérieur, notamment un adénovirus recombinant, et soit un adénovirus naturel ou sauvage (issu d'une contamination accidentelle ou d'une infection opportuniste d'un organisme hôte), soit un fragment de génome adénoviral intégré dans la lignée de complémentation 293 n'est pas négligeable. En effet, il suffit d'un événement de recombinaison pour restaurer la fonction E1 et générer un adénovirus recombinant non-défectif capable de se disséminer dans l'environnement. Il est aussi envisageable qu'un adénovirus naturel sauvage co-infectant la même cellule qu'un adénovirus défectif puisse complémenter ce dernier pour la fonction E1 provoquant une co-dissémination des deux virus. Enfin, certains types de cellules eucaryotes produisent des protéines présentant une activité E1A-like également susceptibles de complémenter partiellement les adénovirus défectifs qui les infectent.

Il est donc souhaitable de disposer de vecteurs adénoviraux performants présentant le minimum de risque, en vue de leur utilisation en thérapie génique pour corriger *in vivo*

des défauts génétiques graves et traiter certaines maladies pour lesquelles on ne dispose pas d'approches thérapeutiques efficaces. C'est de leur obtention que dépend le succès de la thérapie génique appliquée à l'homme.

De plus, il existe des interrogations à propos de l'obtention de la lignée 293. Ces interrogations peuvent être de nature à compromettre l'acceptabilité des produits destinés à un usage humain qui en seront dérivés. Il serait utile de disposer de lignées de complémentation dont l'origine et l'histoire sont exactement connues pour produire des particules d'adénovirus recombinants destinées à un usage humain.

On a maintenant trouvé (1) de nouveaux vecteurs adénoviraux défectifs délétés de certaines régions spécifiques du génome adénoviral et plus adaptés au transfert d'une séquence nucléotidique exogène *in vivo* et (2) de nouvelles lignées de complémentation caractérisées, acceptables d'un point de vue pharmaceutique et donc offrant toutes les caractéristiques de sécurité requises pour la production de produits destinés à un usage humain.

L'intérêt de ces nouveaux vecteurs est qu'ils présentent une capacité de clonage accrue permettant l'insertion d'un ou plusieurs gènes d'intérêt de grande taille et une sécurité d'emploi maximale. Ces mutations délétères rendent ces adénovirus incapables de réplication autonome et de transformation cellulaire et ceci sans altérer leur capacité à transférer et exprimer un gène d'intérêt.

C'est pourquoi la présente invention a pour objet un vecteur adénoviral défectif pour la réplication, capable d'être encapsidé dans une cellule de complémentation, qui dérive du génome d'un adénovirus comprenant de 5' en 3', un ITR 5', une région d'encapsidation, une région E1A, une région E1B, une région E2, une région E3, une région E4 et un ITR 3', par délétion :

(i) de tout ou partie de la région E1A, et de l'intégralité de la partie de la région E1B codant pour les protéines précoces ; ou

(ii) de tout ou partie de la région E1A et de tout ou partie d'au moins une région sélectionnée parmi les régions E2 et E4 ; ou

(iii) de tout ou partie de la région E1A et d'une partie de la région d'encapsidation.

Au sens de la présente invention, l'expression "délétion" ou "dépourvu" se réfère à la suppression d'au moins un nucléotide dans la région ciblée et bien entendu il peut s'agir d'une délétion continue ou discontinue. Par tout ou partie, on entend soit l'intégralité soit une partie seulement de la région considérée. On préfère les délétions qui empêche la production d'au moins un produit d'expression codé par ladite région. Elles peuvent donc se situer dans une région codante ou dans une région régulatrice comme la région promotrice et concerner au moins un nucléotide de manière à détruire le cadre de lecture d'un gène ou rendre une région promotrice non-fonctionnelle. Il peut également s'agir de délétions partielles d'un ou plusieurs gènes de ladite région ou de l'ensemble de la région.

Un vecteur adénoviral selon l'invention est défectif pour la replication mais capable d'être repliqué et encapsidé dans une cellule de complémentation lui fournissant *en trans* le ou les produit(s) pour lesquels il est défectif afin de générer une particule adénovirale (encore désignée adénovirus défectif) incapable de réplication autonome dans une cellule hôte mais néanmoins infectieuse car ayant la capacité de délivrer le vecteur dans une cellule hôte.

Selon une première variante, un vecteur adénoviral selon l'invention dérive du génome d'un adénovirus naturel ou sauvage par délétion de tout ou partie de la région E1A et de la partie de la région E1B comprenant l'intégralité des séquences codant pour les protéines précoces. Selon un mode préféré, elle concerne le promoteur et les séquences codant pour les produits d'expression de la région E1B c'est à dire les protéines précoces et n'inclut pas tout ou partie du signal de terminaison de la transcription qui recouvre les séquences codant pour la protéine tardive IX. S'agissant d'un vecteur adénoviral selon l'invention dérivant d'un adénovirus humain de type 5, ladite délétion comprend au moins les séquences comprises entre les nucléotides 1634 et 3509 du génome adénoviral dont la séquence est telle que divulguée dans la banque de donnée Genebank sous la référence M73260. Cette délétion a pour but de réduire ou supprimer les séquences communes entre un vecteur adénoviral selon l'invention et le fragment de génome adénoviral intégré dans une lignée de complémentation, par exemple la lignée 293. De plus, elle élimine d'un vecteur adénoviral selon l'invention des séquences dont les produits d'expression sont potentiellement oncogènes, du moins en conjonction avec les produits d'expression de la région E1A.

Par ailleurs, un vecteur adénoviral selon l'invention dérive en outre du génome d'un adénovirus naturel ou sauvage par délétion de tout ou partie :

- de la région E3 et/ou

- de la région E2 et/ou
- de la région E4.

Il va de soi qu'un vecteur adénoviral selon l'invention peut comporter une des trois délétions ci-dessus enoncées ou deux d'entre elles selon n'importe quelles combinaisons ou encore l'ensemble des délétions.

Selon un mode particulièrement avantageux, un vecteur adénoviral selon l'invention est délété d'une partie seulement de la région E3 et préférentiellement de la partie qui ne comprend pas les séquences codant pour la protéine gp19kDa. La présence de la séquence codant pour la protéine gp19kDa dans un vecteur adénoviral selon l'invention, permettra aux cellules infectées d'échapper à l'immunosurveillance de l'hôte ; un critère important lorsque le protocole thérapeutique nécessite plusieurs administrations répétées. On choisira, de préférence, de placer les séquences codant pour la gp19kDa sous le contrôle d'éléments appropriés permettant leur expression dans la cellule hôte, à savoir les éléments nécessaires à la transcription desdites séquences en ARNm et la traduction de ce dernier en protéine. Ces éléments comprennent en particulier un promoteur. De tels promoteurs sont bien connus de l'homme de l'art et sont insérés en amont de ladite séquence codante par les techniques conventionnelles du génie génétique. Le promoteur retenu sera, de préférence, un promoteur constitutif non activable par un des produits d'expression de la région E1A. A titre d'exemples, on peut citer le promoteur du gène HMG (Hydroxy-Methyl-Glutaryl coenzyme A réductase), le promoteur précoce du virus SV40 (Simian Virus 40), le LTR (Long Terminal Repeat) du RSV (Rous Sarcoma Virus) ou le promoteur d'un gène PGK (phospho-glycerate kinase) d'eucaryote supérieur.

Par ailleurs, un vecteur adénoviral selon l'invention, peut, de façon optionnelle, être délété de la partie de la région E3 correspondant à la région promotrice, laquelle sera substituée par une région promotrice hétérologue, telles l'une de celles mentionnées ci-dessus.

Selon une deuxième variante, un vecteur adénoviral selon l'invention dérive du génome d'un adénovirus naturel ou sauvage par délétion continue ou discontinue de tout ou partie de la région E1A et de tout ou partie d'au moins la région E2 et/ou E4. Une telle délétion permet d'accroître les possibilités de clonage de gènes d'intérêt. D'autre part, éliminer tout ou partie de la région E4 permet également de réduire ou supprimer des séquences codant pour des produits potentiellement oncogènes.

Comme précédemment, un vecteur adénoviral selon l'invention peut, en outre être dépourvu de tout ou partie des régions E1B et/ou E3 et, en particulier, selon un mode de réalisation tel que mentionné précedemment (comme la délétion de la partie de la région E1B comprenant l'intégralité des séquences codant pour les protéines précoces et de la partie de la région E3 ne codant pas pour la protéine gp19kDa).

Enfin selon une troisième variante, un vecteur adénoviral selon l'invention dérive du génome d'un adénovirus par délétion de tout ou partie de la région E1A et d'une partie de la région d'encapsidation.

Une délétion partielle de la région d'encapsidation permet de réduire notablement la probabilité de dissémination incontrôlée d'un vecteur adénoviral selon l'invention, lorsque ce dernier est en présence d'un adénovirus sauvage. Une telle délétion permet d'affecter ses fonctions d'encapsidation de telle sorte que même en cas de complémentation *en trans* de la fonction défective de celui-ci par un adénovirus sauvage, il ne pourra être encapsidé efficacement par rapport au génome de l'adénovirus sauvage compétiteur.

Les délétions de la région d'encapsidation sera choisies en fonction de 2 critères : une capacité réduite à être encapsidé mais simultanément une efficacité résiduelle compatible avec une production industrielle. En d'autres termes, la fonction d'encapsidation d'un vecteur adénoviral selon l'invention est substantiellement maintenue quoique à un degré moindre. L'atténuation peut être déterminée par les techniques conventionnelles de titrage par infection d'une lignée adéquate et évaluation du nombre de plages de lyse. De telles techniques sont connues de l'homme de l'art. Dans le cadre de l'invention, l'efficacité d'encapsidation est réduite d'un facteur 2 à 50, avantageusement 3 à 20 et de préférence 5 à 10 par rapport à un adénovirus témoin ayant une région d'encapsidation de type sauvage.

Bien entendu, un vecteur adénoviral atténué selon l'invention, peut en outre comprendre au moins une ou une quelconque combinaison des délétions précédemment citées.

Un vecteur adénoviral selon la présente invention dérive du génome d'un adénovirus naturel ou sauvage, avantageusement d'un adénovirus canin, aviaire ou humain, de préférence d'un adénovirus humain de type 2, 3, 4, 5 ou 7 et, de manière tout à fait préférée, d'un adénovirus humain de type 5 (Ad5). Dans ce dernier cas, les délétions du vecteur adénoviral selon l'invention sont indiquées par référence à la position des nucléotides du génome de l'Ad5 spécifiée dans la banque de donnée Genebank sous la référence M73260.

On préfère tout particulièrement un vecteur adénoviral selon l'invention dérivant du génome d'un adénovirus humain de type 5, par délétion :

(i) de l'intégralité de la partie codant pour les protéines précoces de la région E1B et s'étendant du nucléotide 1634 et se terminant au nucléotide 4047 ; et/ou

(ii) de la région E4 s'étendant des nucléotides 32800 à 35826 ; et/ou

(iii) de la partie de la région E3 s'étendant des nucléotides 27871 à 30748 ; et/ou

(iv) de la partie de la région d'encapsidation :

- allant du nucléotide 270 au nucléotide 346, ou
- allant du nucléotide 184 au nucléotide 273, ou
- allant du nucléotide 287 au nucléotide 358.

De préférence, un vecteur adénoviral selon l'invention dérive du génome d'un adénovirus sauvage ou naturel par délétion d'au moins 18 % dudit génome, d'au moins 22 %, d'au moins 25 %, d'au moins 30 %, d'au moins 40 %, d'au moins 50 %, d'au moins 60 %, d'au moins 70 %, d'au moins 80 %, d'au moins 90 % ou encore d'au moins 95 % et notamment de 98,5%.

Selon un mode particulièrement préféré, un vecteur adénoviral selon l'invention dérive du génome d'un adénovirus par délétion de l'ensemble du génome adénoviral à l'exclusion des ITRs 5' et 3' et de tout ou partie de la région d'encapsidation. Selon cette variante, il ne comprend que le minimum de séquences virales afin de limiter les risques de recombinaison, les risques d'oncogénécité et avoir une capacité de clonage maximale. On parlera alors d'un vecteur adénoviral "minimum" dans lequel il sera alors possible d'insérer jusqu'à 30kb de séquence nucléotidique exogène. Un vecteur adénoviral préféré selon l'invention dérive d'un adénovirus humain de type 5 par délétion de la partie du génome viral s'étendant des nucléotides 459 à 35832.

Dans le cadre de la présente invention, un vecteur adénoviral selon l'invention a pour objet le transfert et l'expression d'une séquence nucléotidique exogène dans une cellule hôte. Par "séquence nucléotidique exogène", on entend un acide nucléique qui comprend des séquences codantes et des séquences régulatrices permettant l'expression desdites séquences codantes et dans lequel les séquences codantes sont des séquences qui ne sont normalement pas présentes dans le génome d'un adénovirus. Les séquences régulatrices peuvent être d'origine quelconque. La séquence nucléotidique exogène est introduite dans un vecteur adénoviral selon l'invention par les techniques classiques du génie génétique, entre la région d'encapsidation et l'ITR 3'.

Une séquence nucléotidique exogène peut être constituée d'un ou plusieurs gène(s) d'intérêt et, de manière préférée, d'intérêt thérapeutique. Dans le cadre de la présente invention, un gène d'intérêt peut coder soit pour un ARN anti-sens, soit pour un ARNm qui sera ensuite traduit en protéine d'intérêt. Un gène d'intérêt peut être de type génomique, de type ADN complémentaire (ADNc) ou de type mixte (minigène, dans lequel au moins un intron est délété). Il peut coder pour une protéine mature, un précurseur d'une protéine mature, notamment un précurseur destiné à être secrété et comprenant de ce fait un peptide signal, une protéine chimérique provenant de la fusion de séquence d'origine diverse ou un mutant d'une protéine naturelle présentant des propriétés biologiques améliorées ou modifiées. Un tel mutant peut être obtenu par mutation, délétion, substitution et/ou addition d'un ou plusieurs nucléotide(s) du gène codant pour la protéine naturelle.

Un gène d'intérêt peut être placé sous le contrôle d'éléments appropriés à son expression dans une cellule hôte. Par "éléments appropriés", on entend l'ensemble des éléments nécessaires à sa transcription en ARN (ARN anti-sens ou ARNm) et à la traduction d'un ARNm en protéine. Parmi les éléments nécessaires à la transcription, le promoteur revêt une importance particulière. Il peut s'agir d'un promoteur constitutif ou d'un promoteur régulable et il peut être isolé d'un gène quelconque d'origine eucaryote ou virale et même adénovirale. Alternativement, il peut s'agir du promoteur naturel du gène d'intérêt en question. D'une façon générale, un promoteur en usage dans la présente invention, peut être modifié de manière à contenir des séquences régulatrices. A titre d'exemples, un gène d'intérêt en usage dans la présente invention, est placé sous le contrôle du promoteur des gènes d'immunoglobuline lorsque l'on cherche à cibler son transfert dans des cellules hôtes lymphocytaires. On peut également citer le promoteur du gène TK-HSV-1 (thymidine kinase du virus de l'herpès de type 1) ou encore le promoteur adénoviral MLP, notamment de l'adénovirus humain de type 2, permettant une expression dans un grand nombre de types cellulaires.

Parmi les gènes d'intérêt utilisables dans le cadre de la présente invention, on peut citer :

- les gènes codant pour des cytokines, comme l'interféron alpha, l'interféron gamma, les interleukines ;
- les gènes codant pour des récepteurs membranaires, comme les récepteurs reconnus par des organismes pathogènes (virus, bactéries, ou parasites), de préférence par le virus VIH (Virus de l'Immunodéficience Humain) ;
- les gènes codant pour des facteurs de coagulation, comme le facteur VIII et le facteur IX ;
- le gène codant pour la dystrophine ;
- le gène codant pour l'insuline ;
- les gènes codant pour des protéines participant directement ou indirectement aux canaux ioniques cellulaires, comme la protéine CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) ;
- les gènes codant pour des ARN anti-sens ou des protéines capables d'inhiber l'activité d'une protéine produite par un gène pathogène, présent dans le génome d'un organisme pathogène, ou par un gène cellulaire, dont l'expression est dérégulée, par exemple un oncogène ;
- les gènes codant pour une protéine inhibant une activité enzymatique, comme l'α1- antitrypsine ou un inhibiteur d'une protéase virale ;
- les gènes codant pour des variants de protéines pathogènes qui ont été mutées de façon à altérer leur fonction biologique, comme par exemple des variants trans-dominants de la protéine TAT du virus VIH capables de compétition avec la protéine naturelle pour la liaison à la séquence cible, empêchant ainsi l'activation du VIH ;
- les gènes codant pour des épitopes antigéniques afin d'accroître l'immunité de la cellule hôte ;

- les gènes codant pour les protéines de complexe majeur d'histocompatibilité des classes I et II, ainsi que les gènes codant pour les protéines inductrices de ces gènes ;

- les gènes codant pour des enzymes cellulaires ou produites par des organismes pathogènes ; et

- les gènes suicides. On peut citer plus particulièrement le gène suicide TK-HSV-1. L'enzyme TK virale présente une affinité nettement supérieure par rapport à l'enzyme TK cellulaire pour certains analogues de nucléosides (comme l'acyclovir ou le gancyclovir). Elle les convertit en molécules monophosphatées, elles-mêmes convertibles, par les enzymes cellulaires, en précurseurs de nucléotides, qui sont toxiques. Ces analogues de nucléotides sont incorporables dans les molécules d'ADN en voie de synthèse, donc principalement dans l'ADN des cellules en état de réplication. Cette incorporation permet de détruire spécifiquement les cellules en division comme les cellules cancéreuses.

Cette liste n'est pas limitative et d'autres gènes d'intérêt peuvent être utilisés dans le cadre de la présente invention.

Par ailleurs, selon un autre mode de mise en oeuvre de l'invention, un vecteur adénoviral selon l'invention peut en outre comprendre un gène non-thérapeutique codant pour une protéine trans-activatrice de la transcription non-adénovirale. Bien entendu, on évitera le ou les gène(s) de la région E1A codant pour une protéine trans-activatrice, dont l'expression risquerait de rendre l'adénovirus non-défectif. On choisira, de préférence, le gène codant pour la protéine Gal4 de *Saccharomyces cerevisiae*. Son expression permettra la propagation du vecteur dans une lignée de complémentation telle que celle décrite ci-après. Une telle lignée est plus sophistiquée et permet de pallier à d'éventuels problèmes de toxicité due à la production en continue des protéines adénovirales de complémentation. Le gène codant pour une protéine trans-activatrice de la transcription peut être placé, si nécessaire, sous le contrôle d'éléments appropriés à son expression ; par exemple ceux qui permettent l'expression d'un gène d'intérêt.

L'invention a également trait à une particule adénovirale ainsi qu'à une cellule eucaryote hôte comprenant un vecteur adénoviral selon l'invention. Ladite cellule est avantageusement une cellule de mammifère et, de préférence, une cellule humaine et peut

comprendre ledit vecteur sous forme intégrée dans le génome ou, de préférence, sous forme non-intégrée (épisome).

Une particule adénovirale selon l'invention peut être préparée par passage dans toute lignée de complémentation fournissant *en trans* les fonctions pour lesquelles un vecteur adénoviral selon l'invention est défectif, par exemple la lignée 293 de l'art antérieur. Ces techniques de préparation sont connues de l'homme de l'art (Graham et Prevec, 1991, Methods in Molecular Biology, vol 7, 109-128, Ed : E.J. Murey, The Human Press Inc.). D'une manière optionnelle, une particule adénovirale selon l'invention peut être générée dans une lignée de complémentation selon l'invention telle que décrite ci-après.

C'est pourquoi la présente invention concerne également une lignée de complémentation comportant un élément de complémentation, comprenant notamment une partie de la région E1 du génome d'un adénovirus à l'exclusion de l'ITR 5' ; ledit élément de complémentation étant capable de complémenter *en trans* un vecteur adénoviral défectif et étant intégré dans le génome de ladite lignée de complémentation ou inséré dans un vecteur d'expression.

Dans le cadre de la présente invention, le terme "lignée de complémentation" se réfère à une cellule eucaryote capable de fournir *en trans* la ou les fonction(s) pour la(les)quelle(s) un vecteur adénoviral est défectif. En d'autres termes, elle est capable de produire l'une ou les protéine(s) nécessaire(s) à la replication et à l'encapsidation dudit vecteur adénoviral, protéines précoces et/ou tardives qu'il ne peut lui même produire et qui sont nécessaires à la constitution d'une particule virale. Bien entendu, ladite partie peut être modifiée par mutation, délétion et/ou addition de nucléotides, du moment que ces modifications n'altèrent pas sa capacité de complémentation. Ainsi, un vecteur adénoviral défectif pour la fonction E1 devra être propagé dans une lignée de complémentation pour E1 (capable de fournir *en trans* la ou l'ensemble des protéines codées par la région E1 que le vecteur ne peut produire), un vecteur défectif pour les fonctions E1 et E4 le sera dans une lignée de complémentation pour E1 et E4 (fournissant les protéines nécéssaires codées par les régions E1 et E4) et, enfin, un vecteur défectif pour les fonctions E1, E2 et E4 le sera dans une lignée de complémentation pour les trois fonctions. Comme indiqué dans l'introduction, la région E3 est non essentielle, et ne nécessite pas d'être spécifiquement complétée.

Une lignée de complémentation selon l'invention peut être dérivée soit d'une lignée cellulaire immortalisée, capable de se diviser indéfiniment, soit d'une lignée primaire. Conformément aux buts poursuivis par la présente invention, une lignée de

complémentation selon l'invention est utile pour l'encapsidation de n'importe quel vecteur adénoviral défectif et, en particulier, d'un vecteur adénoviral défectif selon l'invention. Ainsi, lorsqu'on utilisera ci-après le terme "vecteur adénoviral défectif", il doit être entendu qu'il fait référence à un vecteur défectif quelconque, de l'art antérieur ou de la présente invention.

Par "élément de complémentation", on entend un acide nucléique comprenant au moins la partie du génome adénoviral en usage dans le cadre de la présente invention. Il peut être inséré sur un vecteur, par exemple de type plasmidique ou viral, par exemple rétroviral, adénoviral ou dérivé d'un poxvirus. On préférera néanmoins le cas où il est intégré dans le génome d'une lignée de complémentation selon l'invention. Les méthodes pour introduire un vecteur ou un acide nucléique dans une lignée cellulaire et éventuellement l'intégrer dans le génome d'une cellule constituent des techniques conventionnelles bien connues de l'homme de l'art, de même que les vecteurs utilisables à de telles fins. L'élément de complémentation peut être introduit dans une lignée de complémentation selon l'invention, de façon préalable ou concomitante à un vecteur adénoviral défectif.

Selon un mode de réalisation spécifique, une lignée de complémentation selon l'invention est destinée à complémenter *en trans* un vecteur adénoviral défectif pour la fonction E1. Une telle lignée présente l'avantage de diminuer les risques de recombinaison puisque, contrairement à la lignée conventionnelle 293, elle est dépourvue de l'ITR 5' présent dans les vecteurs.

Dans le cadre de la présente invention, une lignée de complémentation selon l'invention peut comprendre tout ou partie de la région E1A du génome d'un adénovirus et :

(i) tout ou partie d'au moins une région du génome adénoviral sélectionnée parmi les régions E1B, E2 et E4, ou

(ii) tout ou partie d'au moins deux des régions E1B, E2 et E4 dudit génome, ou

(iii) tout ou partie des régions E1B, E2 et E4 dudit génome.

Dans le cadre de l'invention, lesdites régions peuvent être placées, si nécessaire, sous le contrôle d'éléments appropriés permettant leur expression. mais, on préfère les placer sous le contrôle de leur propre promoteur, inductible par la protéine trans-activatrice de transcription codée par la région E1A.

A titre indicatif, une lignée de complémentation selon la variante (ii) comprenant les régions E1A, E1B et E4 est destinée à la préparation d'un adénovirus défectif pour les fonctions E1 et E4 délété de tout ou partie des régions correspondantes.

Selon un mode avantageux, une lignée de complémentation selon l'invention, comprend notamment tout ou partie de la région E1A et l'intégralité des séquences codant pour les protéines précoces de la région E1B.

Par ailleurs, selon une variante de ce mode de réalisation, une lignée de complémentation selon l'invention peut, en outre, être dépourvue de la région promotrice de la région E1A. Dans ce cas, la partie du génome adénoviral codant pour les protéines précoces de ladite région E1A sera placée sous le contrôle d'un promoteur hétérologue approprié et fonctionnel dans ladite lignée de complémentation. Il peut être isolé de n'importe quel gène eucaryote ou viral. On évitera, cependant, d'avoir recours à un promoteur adénoviral d'une région précoce. Il peut s'agir d'un promoteur constitutif. A titre d'exemples, on peut citer les promoteurs du virus SV40, du gène TK-HSV-1 et du gène murin PGK.

D'une manière alternative, le promoteur retenu peut être régulable et avantageusement, inductible par une protéine trans-activatrice de transcription non adénovirale. Il peut s'agir d'un promoteur isolé d'un gène naturellement inductible ou d'un promoteur quelconque modifié par l'addition de séquences d'activation (ou UAS, pour Upstream Activating Sequence en anglais) répondant à ladite protéine trans-activatrice. De manière plus particulière, on préfère utiliser un promoteur inductible par la protéine Gal4 de *Saccharomyces cerevisiae* et, de préférence, un promoteur hybride constitué d'un promoteur dit "minimum" contenant uniquement les séquences d'initiation de la transcription (TATA box et site d'initiation) d'un gène quelconque (par exemple du gène TK-HSV-1 ou MLP d'Ad2), en amont duquel on a inséré au moins une séquence d'activation du gène Gal10 de *Saccharomyces cerevisiae* (Webster et al., 1988, Cell, *52*, 169-178). Cette dernière peut être synthétisée chimiquement ou isolée du gène Gal10, selon les techniques classiques du génie génétique. Ainsi, le promoteur hybride ne sera activé et n'induira l'expression des gènes codés par la région E1A placés sous son contrôle, qu'en présence de la protéine Gal4. Puis, les produits d'expression de la région E1A pourront à leur tour, induire l'expression des autres régions précoces E1B, E2 et/ou E4 éventuellement comprises dans une lignée de complémentation selon l'invention. Ce mode de réalisation particulier de l'invention, évite la production d'une manière constitutive (éventuellement toxique) des protéines adénovirales nécessaires à la

complémentation. Ainsi, l'induction peut être déclenchée en présence d'un vecteur adénoviral défectif selon l'invention exprimant la protéine Gal4. Cependant une telle lignée peut également être utilisée pour préparer n'importe quel vecteur adénoviral défectif, à la condition toutefois de fournir *en trans* la protéine Gal4. Les moyens de fournir *en trans* une protéine sont connus de l'homme du métier.

D'une manière générale, une lignée de complémentation comprend une partie du génome d'un adénovirus qui dérive avantageusement d'un adénovirus animal, comme un adénovirus canin ou aviaire ou, de préférence, d'un adénovirus humain et, tout particulièrement, du type 2 ou 5.

Une lignée de complémentation selon l'invention comprend notamment la partie du génome d'un adénovirus humain de type 5 s'étendant :

(i) du nucléotide 100 au nucléotide 5297 de la séquence telle que divulguée dans la banque de donnée Genebank sous la référence M73260, ou

(ii) du nucléotide 100 au nucléotide 4034, ou

(iii) du nucléotide 505 au nucléotide 4034.

Avantageusement, la partie du génome selon (ii) est insérée en amont d'un signal de terminaison de la transcription, comme par exemple le signal de polyadénylation du virus SV40 (Simian Virus 40) ou du gène ß-globine de lapin. Alors que la partie selon (iii) qui ne comprend ni les séquences promotrices de la région E1A, ni le signal de terminaison de la transcription de la région E1B est placée sous le contrôle d'un promoteur approprié, notamment d'un promoteur inductible par la protéine Gal4, et d'un signal de terminaison de la transcription, par exemple celui du gène ß-globine de lapin. Une telle lignée de complémentation est considérée comme particulièrement sûre car dépourvue de la majorité des séquences communes avec un adénovirus défectif.

D'autre part, une lignée de complémentation selon l'invention peut comporter la partie de la région E4 d'un adénovirus humain de type 5 allant du nucléotide 32800 et se terminant au nucléotide 35826 de la séquence telle que divulguée dans la banque de donnée Genebank sous la référence M73260.

Par ailleurs, une lignée de complémentation selon l'invention peut comporter l'ensemble du génome d'un adénovirus naturel, à l'exception de la région d'encapsidation et des ITRs

5' et 3' et, de manière tout à fait préférée, la partie du génome d'un adénovirus humain de type 5 allant du nucléotide 505 et se terminant au nucléotide 35826 de la séquence telle que divulguée dans la banque de donnée Genebank sous la référence M73260. Aux fins de la présente invention, celle-ci est placée sous le contrôle d'un promoteur approprié. On aura, de préférence, recours à un promoteur inductible par la protéine Gal4 de *Saccharomyces cerevisae*. Une telle lignée permettra de complémenter *en trans* l'ensemble des fonctions essentielles à la replication et l'encapsidation d'un vecteur adénoviral défectif pour les fonctions E1, E2 et E4, notamment d'un vecteur adénoviral minimum selon l'invention.

Selon un mode préféré, une lignée de complémentation selon l'invention peut comporter un élément de complémentation comprenant, en outre, un gène codant pour un marqueur de sélection permettant la détection et l'isolement des cellules le comportant. Dans le contexte de la présente invention, il peut s'agir de n'importe quel gène codant pour un marqueur de sélection, ceux-ci étant généralement connus de l'homme de l'art, avantageusement d'un gène de résistance à un antibiotique et, de préférence, du gène codant pour la puromycine acetyl-transférase (gène pac) conférant la résistance à la puromycine.

Dans le cadre de la présente invention, le gène codant pour un marqueur de sélection peut être placé sous le contrôle des éléments appropriés permettant son expression. Il peut s'agir d'un promoteur constitutif, comme le promoteur précoce du virus SV40. Cependant, on préférera un promoteur inductible par la protéine trans-activatrice codée par la région E1A, en particulier le promoteur adénoviral E2A. Une telle combinaison introduira une pression de selection pour maintenir l'expression des gènes de la région E1A dans une lignée de complémentation selon l'invention. Aux fins de la présente invention, le promoteur retenu peut être modifié par délétion, mutation, substitution et/ou addition de nucléotides.

Selon un mode de réalisation tout à fait préféré, une lignée de complémentation selon l'invention est dérivée d'une lignée cellulaire acceptable d'un point de vue pharmaceutique. Par "lignée cellulaire acceptable d'un point de vue pharmaceutique", on entend une lignée cellulaire caractérisée (dont on connait l'origine et l'histoire) et/ou ayant déjà été utilisée pour la production à grande échelle de produits destinés à un usage humain (constitution de lots pour des essais cliniques avancés ou de lots destinés à la vente). De telles lignées sont disponibles dans des organismes tels que l'ATCC. A cet égard, on peut mentionner les lignées de rein de singe vert d'Afrique Vero, de rein de hamster doré ou syrien BHK, humaine dérivée d'un carcinome de poumon A549,

humaine pulmonaire MRC5, humaine pulmonaire W138 et d'ovaire de hamster chinois CHO.

D'une manière alternative, une lignée de complémentation selon l'invention peut dériver de cellules primaires et notamment de cellules de rétine prélevées d'un embryon humain.

L'invention concerne également un procédé de préparation d'une particule adénovirale selon l'invention, selon lequel :

- on introduit un vecteur adénoviral selon l'invention dans une lignée de complémentation capable de complémenter *en trans* ledit vecteur, de manière à obtenir une lignée de complémentation transfectée,

- on cultive ladite lignée de complémentation selon des conditions appropriées pour permettre la production de ladite particule adénovirale, et

- on récupère ladite particule dans la culture cellulaire.

Bien entendu, la particule adénovirale peut être récupérée du surnageant de culture mais, également des cellules selon les protocoles conventionnels.

D'une manière préférée, un procédé selon l'invention met en oeuvre une lignée de complémentation selon l'invention.

L'invention a également pour objet l'usage thérapeutique ou prophylactique d'un vecteur adénoviral, d'une particule d'adénovirus, d'une cellule eucaryote hôte ou d'une lignée de complémentation selon l'invention.

La présente invention est enfin relative à une composition pharmaceutique comprenant à titre d'agent thérapeutique ou prophylactique un vecteur adénoviral, une particule d'adénovirus, une cellule eucaryote ou une cellule de complémentation selon l'invention, en association avec un support acceptable d'un point de vue pharmaceutique.

La composition selon l'invention, est en particulier destinée au traitement préventif ou curatif de maladies telles que :

- des maladies génétiques, comme l'hémophilie, la mucoviscidose ou la myopathie, celle de Duchêne et de Becker,

- des cancers, comme ceux induits par des oncogènes ou des virus,

- des maladies rétrovirales, comme le SIDA (syndrome de l'immunodéficience acquise résultant de l'infection par le VIH), et

- des maladies virales récurrentes, comme les infections virales provoquées par le virus de l'herpès.

Une composition pharmaceutique selon l'invention peut être fabriquée de manière conventionnelle. En particulier, on associe une quantité thérapeutiquement efficace d'un agent thérapeutique ou prophylactique à un support tel qu'un diluant. Une composition selon l'invention peut être administrée par aérosol ou par n'importe quelle voie conventionnelle en usage dans le domaine de l'art, en particulier par voie orale, sous-cutanée, intramusculaire, intraveineuse, intrapéritonéale intrapulmonaire ou intra-trachéale. L'administration peut avoir lieu en dose unique ou répétée une ou plusieurs fois après un certain délai d'intervalle. La voie d'administration et le dosage appropriés varient en fonction de divers paramètres, par exemple, de l'individu traité ou de la maladie à traiter ou encore du ou des gène(s) d'intérêt à transférer. D'une manière générale, une composition pharmaceutique selon l'invention comprend une dose d'adénovirus selon l'invention comprise entre 10^4 et 10^{14}, avantageusement 10^5 et 10^{13} et de préférence 10^6 et 10^{11}. Une composition pharmaceutique, en particulier à visée prophylactique, peut comprendre en outre un adjuvant acceptable d'un point de vue pharmaceutique.

L'invention s'étend également à une méthode de traitement selon laquelle on administre une quantité thérapeutiquement efficace d'un vecteur adénoviral, d'une particule adénovirale, d'une cellule eucaryote ou d'une lignée de complémentation selon l'invention à un patient ayant besoin d'un tel traitement.

La présente invention est plus complètement décrite en référence aux Figures suivantes et à l'aide des exemples suivants.

La Figure 1 est une représentation schématique du génome de l'adénovirus humain de type 5 (représenté en unités arbitraires de 0 à 100), indiquant l'emplacement des différents gènes.

La Figure 2 est une représentation schématique du vecteur pTG6546.

La Figure 3 est une représentation schématique du vecteur pTG6581.

La Figure 4 est une représentation schématique du vecteur pTG6303.

La Figure 5 est une représentation schématique des vecteurs pTG1660 et pTG1661.

La Figure 6 est une représentation schématique des vecteurs pTG1653, pTG1654 et pTG1655.

La Figure 7 est une représentation schématique du vecteur pTG5913.

La Figure 8 est une représentation schématique du vecteur pTG8512.

La Figure 9 est une représentation schématique du vecteur pTG8513.

La Figure 10 est une représentation schématique du vecteur pTG8514.

La Figure 11 est une représentation schématique du vecteur pTG8515.

EXEMPLES

Les exemples suivants n'illustrent qu'un mode de réalisation de la présente invention.

Les constructions décrites ci-dessous sont réalisées selon les techniques générales de génie génétique et de clonage moléculaire, détaillées dans Maniatis et al., (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY). L'ensemble des étapes de clonage mettant en oeuvre des plasmides bactériens est réalisé par passage dans la souche *Escherichia coli (E. coli)* 5K ou BJ, alors que celles qui mettent en oeuvre des vecteurs dérivés du phage M13 sont réalisées par passage dans *E. coli* NM 522. En ce qui concerne les étapes d'amplification par PCR, on applique le protocole tel que décrit dans PCR Protocols-A guide to methods and applications, (1990, edité par Innis, Gelfand, Sninsky and White, Academic Press Inc.).

D'autre part, les cellules sont transfectées selon les techniques standards bien connues de l'homme du métier. On peut citer la technique au phosphate de calcium (Maniatis et al.,

supra). Mais d'autres protocoles permettant d'introduire un acide nucléique dans une cellule peuvent également être employés, tels que la technique au DEAE dextran, l'électroporation, les méthodes basées sur les chocs osmotiques, la microinjection d'une cellule sélectionnée ou les méthodes basées sur l'emploi de liposomes.

Les fragments insérés dans les différentes constructions décrites ci-après, sont indiqués précisément selon leur position dans la séquence nucléotidique :

- du génome de l'Ad5 telle que divulguée dans la banque de données Genebank sous la référence M73260,

- du génome de l'adénovirus de type 2 (Ad2), telle que divulguée dans la banque de données Genebank sous la référence J01949,

- du génome du virus SV40 telle que divulguée dans la banque de données Genebank sous la référence J02400.

EXEMPLE 1 : Génération d'un adénovirus "atténué" comprenant une délétion d'une partie de la région d'encapsidation.

1. Construction d'un vecteur "atténué" comprenant une délétion du nucléotide 184 au nucléotide 273 de la région d'encapsidation

On construit un vecteur comprenant

- l'ITR 5' du génome de l'Ad5 (du nucléotide 1 au nucléotide 103),

- la région d'encapsidation de l'Ad5 comprise entre les nucléotides 104 à 458 dans laquelle la portion allant du nucléotide 184 au nucléotide 273 est délétée et la thymine (T) en position 176 est modifiée en une cytosine (C) afin de créer un site de restriction *Aat*II,

- une cassette d'expression d'un gène d'intérêt comprenant de 5' vers 3' le MLP de l'Ad2 (nucléotides 5779 à 6038), les sites de restriction *Kpn*I-*Xba*I-*Hind*III et *Bam*HI, l'ADNc humain codant pour la protéine CFTR, (la composition en acides aminés correspond à la séquence publiée par Riordan et al, 1989, Science, *245*, 1066-1073 ; à l'exception d'une valine à la place de la méthionine en position 470),

les sites *Pst*I, *Xho*I et *Sal*I et enfin le signal de terminaison de la transcription du virus SV40 (nucléotides 2665 à 2538), et

- le fragment du génome de l'Ad5 s'étendant du nucléotide 3329 au nucléotide 6241.

Dans un premier temps, on clone entre les sites *Eco*RI et *Eco*RV du vecteur M13TG131 (Kieny et al., 1983, Gene, *26*, 91-99) le fragment *Eco*RI *Sma*I isolé de pMLP11. Cette construction est issue de pMLP10 (Levrero et al., 1991, Gene, *101*, 195-202) et diffère du vecteur parental par l'introduction d'un site *Sma*I au niveau du site *Hind*III. On obtient le vecteur M13TG6501. Celui-ci est soumis à une mutagénèse dirigée afin de déléter les séquences comprises entre les nucléotides 184 à 273 de la région d'encapsidation. La mutagénèse dirigée est réalisée à l'aide d'un kit commercial (Amersham) selon les recommandations du fournisseur, et met en oeuvre l'oligonucléotide OTG4174 reporté dans l'identificateur de séquence n°1 (SEQ ID NO: 1). Le vecteur muté est désigné M13TG6502. La région d'encapsidation ainsi délétée est réintroduite sous forme d'un fragment *Eco*RI-*Bgl*II, le site *Bgl*II étant rendu franc par traitement à l'ADN polymérase Klenow, dans le vecteur pMLP11 digéré par *Eco*RI et *Sma*I.

Le vecteur obtenu, pTG6500, est digéré partiellement par *Pst*I, traité à l'ADN polymérase du phage T4 puis digéré par *Pvu*I. On insère dans ce vecteur le fragment *Pvu*I-*Hpa*I isolé de pTG5955 (dérivé de pMLP11). Ce fragment comporte le signal de terminaison de la transcription du virus SV40 et la partie du génome de l'Ad5 s'étendant du nucléotide 3329 au nucléotide 6241. Le vecteur pTG6505 ainsi généré est digéré partiellement par *Sph*I, traité à l'ADN polymérase du phage T4 et religué, ceci afin de détruire le site *Sph*I situé en 5' du polylinker. Il résulte le pTG6511, dans lequel on clone, après digestion par *Bam*HI et traitement à l'ADN polymérase Klenow, l'ADNc CFTR humain sous forme d'un fragment aux extrémités franches généré par digestion *Xho*I et *Ava*I et traitement à l'ADN polymérase Klenow. On obtient pTG6525. A titre indicatif, l'ADNc CFTR est isolé d'un plasmide de l'art antérieur, tel que pTG5960 (Dalemans et al., 1991, Nature, *354*, 526-528).

2. *Construction d'un vecteur "atténué" comprenant une délétion du nucléotide 270 au nucléotide 346 de la région d'encapsidation*.

Le vecteur M13TG6501 est soumis à une mutagénèse dirigée mettant en oeuvre l'oligonucléotide OTG4173 (SEQ ID NO: 2). Puis le fragment muté est réintroduit dans pMLP11, comme indiqué précédemment, pour générer le vecteur pTG6501. Ce dernier

est digéré par *Sph*I, traité à l'ADN polymérase du phage T4, puis par *Pvu*I. On obtient pTG6546 (Figure 2) par clonage du fragment *Pvu*I-*Kpn*I (le site *Kpn*I ayant été rendu franc) isolé de pTG6525 et comportant l'ADNc CFTR humain.

3. *Construction d'un vecteur "atténué" comprenant une délétion du nucléotide 287 au nucléotide 358 de la région d'encapsidation.*

Le vecteur M13TG6501 est soumis à une mutagénèse dirigée afin de déléter les séquences comprises entre les nucléotides 287 et 358 de la région d'encapsidation et de modifier les thymines en position 275 et 276 en guanines pour introduire un site *Nco*I. La mutagénèse est réalisée à l'aide de l'oligonucléotide OTG4191 (SEQ ID NO: 3) pour donner M13TG6507. Ce dernier est clivé par *Bgl*II, traité à l'ADN polymérase Klenow puis digéré par *Eco*RI et on purifie le fragment correspondant muté que l'on introduit dans pMLP11 digéré par *Eco*RI et *Sma*I. On génère pTG6504, duquel on isole le fragment *Sph*I (site rendu franc par traitement à l'ADN polymérase du phage T4)-*Pvu*I que l'on insère entre les sites *Kpn*I (rendu franc par traitement à la T4 polymérase) et *Pvu*I de pTG6511. On obtient pTG6513 qui est traité par *Bam*HI et l'ADN polymérase Klenow avant d'insérer le fragment *Ava*I et *Xho*I de pTG5960 pour donner pTG6526.

4. *Génération d'un adénovirus recombinant défectif et atténué.*

Les adénovirus défectifs recombinants sont générés par co-transfection dans les cellules 293 de soit pTG6525, pTG6526 ou pTG6546 linéarisé par *Cla*I et d'ADN génomique de l'Ad-dl324 (Thimmappaya et al., 1982, Cell, *31*, 543-551) également digéré par *Cla*I, de manière à générer un virus recombinant par recombinaison homologue. Après 8 à 10 jours, les plages individuelles sont isolées, amplifiées dans les cellules 293 et analysées par cartographie de restriction. Des stocks viraux (AdTG6525, AdTG6526 et AdTG6546) sont constitués et leur titre déterminé selon les techniques conventionnelles.

Le virus AdTG6546 est placé en situation de compétition par co-infection avec l'Ad-CFTR (Rosenfeld et al., 1992, Cell, *68*, 143-155) qui comporte une région d'encapsidation de type sauvage. On infecte les cellules 293 par 5 ufp (unité formant des plages) d'Ad-CFTR et 5 ufp d'AdTG6546 par cellule. On isole en parallèle l'ADN viral total par la méthode de Hirt (Gluzman et Van Doren,1983, J. Virol., *45*, 91-103) et l'ADN viral encapsidé après traitement des cellules avec 0,2 % déoxydrolate puis avec 10 µg/ml de déoxyribonucléase (DNase) I, pour éliminer les ADN non protégés dans les virions. Alors que la quantité d'ADN total d'Ad-CFTR et d'AdTG6546 est identique, il y a environ 3 fois moins d'ADN d'AdTG6546 que d'ADN d'Ad-CFTR encapsidé.

On mesure le niveau d'expression de la protéine CFTR dans les extraits cellulaires de cellules 293 infectées par AdTG6546. L'analyse est effectuée par Western blot selon la technique décrite dans Dalemans et al. (1991, Nature, *supra*) en mettant en oeuvre l'anticorps monoclonal MATG1031. Mais, tout autre anticorps reconnaissant des épitopes antigéniques de la protéine CFTR peut être utilisé. On révèle un produit d'une masse moléculaire attendue d'environ 170 kDa. A titre indicatif, le niveau de production est à peu près équivalent à celui obtenu dans les extraits cellulaires infectés par le virus non atténué Ad-CFTR.

EXEMPLE 2 : Génération d'un adénovirus défectif délété de la région E1A et de l'intégralité des séquences codant pour les protéines précoces de la région E1B..

1. Obtention d'un adénovirus recombinant pour l'expression de la protéine CFTR (AdTG6581)

Un tel adénovirus est généré à partir d'un vecteur plasmidique pTG6581 comprenant de 5' vers 3' :

- l'ITR 5' de l'Ad5 (des nucléotides 1à 103),
- la région d'encapsidation de l'Ad5 (des nucléotides 104 à 458),
- une séquence nucléotidique exogène comportant une cassette d'expression, laquelle comprend les éléments suivants :
  - * le MLP de l'Ad2 (nucléotides 5779 à 6038), suivi des trois leaders tripartites également de l'Ad2 (nucléotides 6039-6079 ; nucléotides 7101-7175 ; nucléotides 9637-9712) ; ces leaders sont inclus afin d'augmenter l'efficacité de traduction des séquences insérées en aval,
  - * un polylinker comprenant de 5' vers 3' les sites de restrictions *Xba*I, *Hind*III, *Bam*HI *Eco*RV, *Hpa*I et *Not*I utilisables pour le clonage d'un gène d'intérêt,
  - * un gène d'intérêt, comme le gène codant pour la protéine CFTR,
  - * le signal de terminaison de la transcription isolé du virus SV40 (nucléotides 2543 à 2618),

- la portion du génome adénoviral de l'Ad5 allant des nucléotides 4047 à 6241.

Le fragment du génome de l'Ad5 s'étendant du nucléotide 4047 au nucléotide 4614 est amplifié par PCR à partir de l'ADN génomique d'Ad5. La réaction PCR met en oeuvre l'amorce sens OTG5021 (SEQ ID NO: 4), comprenant en son extrémité 5' un site *Bam*HI destiné à faciliter les étapes de clonage ultérieures, et l'amorce anti-sens OTG5157 (SEQ ID NO: 5.) Le fragment ainsi généré est traité à l'ADN polymérase Klenow, avant d'être cloné dans le site *Sma*I de M13mp18 (Gibco BRL), donnant lieu au M13TG6517. La séquence du fragment généré par PCR est vérifiée selon la méthode enzymatique classique (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, *74*, 5463).

Par ailleurs, le fragment *Pvu*I-*Sma*I est isolé de pMLP11. Il est cloné entre les sites *Pvu*I et *Kpn*I de pTG6511 (exemple 1.1), le site *Kpn*I ayant été rendu franc par un traitement à l'ADN polymérase du phage T4 selon les méthodes standards. On génère ainsi le vecteur pTG6547.

Ce dernier est digéré par les enzymes *Sal*I et *Bst*XI et ligué à deux fragments, d'une part le fragment *Bam*HI-*Bst*XI purifié de M13TG6517 et, d'autre part, le fragment *Xho*I-*Bgl*II de pTG6185. Ce dernier comprend notamment le signal de terminaison de la transcription du virus SV40 encadré par les sites de restriction *Xho*I et *Bgl*II. Mais, tout autre plasmide comportant la même séquence de terminaison et des sites de restriction adéquates pourrait être utilisé. On obtient le vecteur pTG6555, dans lequel on insère dans le site unique *Bam*HI un adaptateur contenant deux sites de restriction générant des extrémités franches, *Eco*RV et *Hpa*I. Cet adaptateur provient de la réassociation des oligonucléotides OTG5564 et OTG5565 (SEQ ID NO: 6 et 7). On obtient pTG6580. Enfin, le fragment *Sac*I-*Pst*I de pTG6525 dont les extrémités ont été rendues franches et comportant l'ADNc CFTR humain, est cloné dans le site *Eco*RV de pTG6580. On génère pTG6581 (Figure 3).

L'adénovirus recombinant correspondant AdTG6581 est généré par co-transfection de pTG6581 et Ad dl324 clivés par *Cla*I dans une lignée de complémentation pour la fonction E1, comme la lignée 293 ou une lignée de l'exemple 6, selon le protocole classique.

2. *Obtention d'un adénovirus recombinant pour l'expression de l'IFNγ.*

Le vecteur pTG6303 (Figure 4) est obtenu par clonage dans le site *Hpa*I de pTG6580 du fragment *Hpa*I-*Sma*I de M13TG2437. Ce dernier provient du clonage dans un vecteur M13TG130 (Kieny et al., 1983, *supra*) du gène codant pour l'interféron gamma (IFNγ) dont la séquence est telle que spécifiée dans Gray et al. (1982, Nature, *295*, 503-508). L'adénovirus recombinant AdTG6303 est obtenu selon les techniques classiques par recombinaison homologue résultant de la co-transfection de pTG6303 et de l'Ad dl324 linéarisé par *Cla*I dans une lignée de complémentation pour la fonction E1.

3. *Construction d'un adénovirus délété de la région E1 et dans lequel la région E3 est placée sous le contrôle d'un promoteur constitutif.*

Le vecteur pTG1670 est obtenu par clonage entre les sites *Aat*II et *Bam*HI du vecteur p polyII (Lathe et al., 1987, Gene *57*, 193-201), d'un fragment PCR comportant le LTR3' (Long Terminal Repeat) du virus RSV (Rous Sarcoma Virus). La réaction PCR met en oeuvre le vecteur pRSV/L (De Wet et al., 1987, Mol. Cell. Biol. *7*, 725-737) à titre de matrice et les amorces OTG5892 et OTG5893 (SEQ ID NO: 8 et 9).

Par ailleurs, la partie 5' de la région E3 (nucléotides 27588 à 28607) est amplifiée par PCR à partir du vecteur pTG1659 et à l'aide des amorces OTG5920 et OTG5891 (SEQ ID NO: 10 et 11). Ce dernier est construit en plusieurs étapes. Le fragment *Bam*HI-*Avr*II (nucléotides 21562 à 28752) est obtenu de l'ADN génomique d'Ad5 puis cloné entre les mêmes sites de pTG7457 pour générer pTG1649. Le vecteur pTG7457 est un pUC19 (Gibco BRL) modifié au niveau du polylinker de manière à contenir notamment un site *Avr*II. Puis on introduit le fragment *Eco*RI (Klenow)-*Avr*II de M13TG1646 (exemple 8) dans pTG1649 clivé par *Avr*II-*Nde*I (Klenow), ce qui donne le vecteur pTG1651. Enfin, pTG1659 est généré par l'insertion du fragment *Avr*II (nucléotides 28752 à 35463) purifié de l'ADN génomique d'Ad5 dans pTG1651 linéarisé par *Avr*II. Le fragment PCR est intégré entre les sites X*ba*I et *Bam*HI et de p poly II, pour donner pTG1671. On insère ensuite dans le site *Aat*II de ce dernier, un fragment *Eco*RV-*Aat*II obtenu de pTG1670, pour donner pTG1676.

Le fragment *Eco*RI de l'Ad5 correspondant aux nucléotides 27331 à 30049, est isolé à partir d'une préparation d'ADN génomique et sous-cloné dans le pBluescript-Sk+ (Stratagène) préalablement clivé par *Eco*RI. On obtient pTG1669. Celui-ci est muté (kit Amersham) par introduction d'un site *Bam*HI soit en position 27867 (oligonucléotide mutagène OTG6079 ; SEQ ID NO: 12) ou en position 28249 (oligonucléotide mutagène OTG6080 ; SEQ ID NO: 13). On obtient respectivement pTG1672 et pTG1673. On isole du vecteur pTG1676, le fragment *Bam*HI-*Bsi*WI comportant le LTR 3' de RSV

suivi de la partie 5' de la région E3 et on l'insère entre les sites *Bam*HI (position 27331 ou 30049) et *Bsi*W (position 28390) des vecteurs obtenus à l'étape précédente pour générer pTG1977 et pTG1978. Puis le fragment *Eco*RI obtenu de chacun de ces deux vecteurs est intègré dans pTG1679, en remplacement du fragment *Eco*RI sauvage. On obtient pTG1679-E3+. A titre indicatif, le vecteur pTG1679 résulte du clonage du fragment *Bst*EII-*Kpn*I (site rendu franc par traitement à la T4 polymerase) de pTG6590 (exemple 3.1) entre les sites *Bst*EII-*Bam*HI (site rendu franc par traitement à la Klenow polymérase) de pTG6584 (exemple 3.1).

On génère une particule d'adénovirus par recombinaison homologue dans une lignée de complémentation pour la fonction E1, entre le fragment *Aat*II de pTG1679-E3+ et un vecteur adénoviral tel que l'Ad dl324 ou Ad-RSVβ-gal. Ce dernier contient le gène de la β-galactosidase à la place de la région E1 (Stratford-Perricaudet et al., 1992, J. Clin. Invest., *90*, 626-630).

EXEMPLE 3 : Construction d'un vecteur adénoviral recombinant à capacité de clonage améliorée par délétion partielle des régions E1 et E3

1. *Construction de pTG6590ΔE3*

Le fragment portant la partie du génome de l'Ad5 comprise entre les nucléotides 27325 et 27871, est amplifié par PCR à partir d'une préparation d'ADN génomique d'Ad5 et à l'aide des amorces OTG6064 et OTG6065 (SEQ ID NO: 14 et 15). OTG6065 comprend à son extrémité 5' un site *Bsm*I, également présent dans la région E3 (en position 30750).

Le fragment amplifié est cloné dans le site *Sma*I de M13mp18, pour donner M13TG6523. Le fragment *Eco*RI-*Bsm*I est isolé de ce dernier pour être introduit dans le vecteur pTG6590 clivé par les mêmes enzymes. On obtient pTG6590Δ3, lequel contient la partie 3' du génome adénoviral (des nucléotides 27082 à 35935) délétée de la région E3 comprise entre les nucléotides 27872 à 30740, alors que pTG6590 est délété d'une partie plus petite de la région E3 (position 28592 à 30470). Le vecteur pTG6590 est obtenu de la façon suivante : on génère par PCR un fragment s'étendant des nucléotides 35228 à 35935 (comportant l'ITR 3') à partir d'une préparation génomique d'Ad5 et à l'aide des amorces OTG5481 et OTG5482 (SEQ ID NO: 16 et 17). Celui-ci est, ensuite, cloné dans le site *Sma*I de M13mp18 pour donner M13TG6519. D'autre part, le vecteur pTG6584 est digéré par *Xba*I puis religué afin d'éliminer le fragment correspondant de la région E3. On obtient pTG6589, lequel est clivé par *Bam*HI, traité à la Klenow puis

digéré par *Bst*EII. On introduit dans le vecteur ainsi traité le fragment *Eco*RI (Klenow)-*Bst*EII purifié de M13TG6519, pour générer pTG6590.

A titre indicatif, le vecteur pTG6584 est un vecteur pUC19 (Gibco BRL) qui contient les séquences d'Ad5 s'étendant du site unique *Spe*I (position 27082) jusqu'au début de la région promotrice de la région E4 (position 35826). Il est obtenu par digestion de pTG1659 (exemple 2.3) par *Sal*I et *Spe*I, traité à l'ADN polymérase Klenow puis religation.

2. *Construction d'un vecteur adénoviral délété de la région E1 et de la partie de E3 n'exprimant pas la protéine gp19kDa*

La partie de la région E3 de l'Ad5 codant pour la gp19kDa (nucléotides 28731 à 29217) est obtenue par PCR à partir d'une préparation d'ADN génomique d'Ad5 et en mettant en oeuvre les amorces OTG5455 et OTG5456 (SEQ ID NO: 18 et 19). Le fragment généré est introduit dans le site *Sma*I de M13mp18 pour donner M13TG6520. On isole le fragment *Eco*RI-*Xba*I de ce dernier, que l'on clone dans le site *Aat*II de pTG1670 (exemple 2.3), les sites ayant été rendus francs par traitement à l'ADN polymérase Klenow. Puis, le fragment *Xba*I purifié du vecteur de l'étape précédente est inséré dans le site *Xba*I du vecteur pTG6590ΔE3 (exemple 3.1.).

3. *Obtention de particules adénovirales.*

Les particules virales recombinantes sont obtenues par ligation des fragments *Spe*I isolés de l'ADN génomique d'AdTG6303 ou AdTG6581 et de l'un ou l'autre des vecteurs des exemples 3.1 et 3.2. Puis, le mélange de ligation est transfecté dans une lignée de complémentation pour la fonction E1.

EXEMPLE 4 : Construction d'un adénovirus délété des régions E1 et E4.

On amplifie les parties du génome adénoviral s'étendant des nucléotides 31803 à 32799 et 35827 à 35935 à partir d'une préparation d'ADN génomique d'Ad5 et des amorces OTG5728 et OTG5729 (SEQ ID NO: 20 et 21) et OTG5730 et OTG5481 (SEQ ID NO: 22 et 16) respectivement. Après une dizaine de cycles d'amplification, la réaction est poursuivie à partir d'une aliquote des deux mélanges réactionnels en mettant en oeuvre les oligonucléotides OTG5728 et OTG5781. Le fragment amplifié s'étend des nucléotides 31803 à 35935 avec une délétion de l'intégralité de la région E4 (positions

32800 à 35826). Après digestion par *Eco*RI et *Hind*III, il est cloné entre les mêmes sites de M13mp18 pour donner M13TG6521.

M13TG6521 est digéré par *Eco*RI, traité à l'ADN polymérase klenow puis clivé par *Bst*XI. Le fragment de 0,46 kb comportant l'ITR 3' est inséré entre le site *Bam*HI rendu franc par traitement à l'ADN polymérase klenow et le site *Bst*XI de pTG6584 (exemple 3.1). On obtient pTG6587, qui est digéré par *Xba*I puis religué sur lui-même, pour donner pTG6588 (délétion de E3).

On introduit dans le site *Pac*I de pTG6588 un fragment d'ADN synthétique provenant de la réassociation des oligonucléotides OTG6060, OTG6061, OTG6062 et OTG6063 (SEQ ID N0: 23 à 26). Il résulte pTG8500 dans lequel les signaux de terminaison de la transcription des gènes tardifs L5 sont améliorés.

On génère une particule adénovirale (AdΔE4) dont le génome est délété de l'intégralité de la région E4 (nucléotides 32800 à 35826) et du fragment *Xba*I de la région E3 (nucléotides 28592 à 30470), par ligation des fragments *Spe*I isolés de pTG8500 ou pTG6588 et de l'Ad5. Le mélange de ligation est transfecté dans une lignée cellulaire de complémentation pour la fonction E4, par exemple la lignée W162 (Weinberg et Ketner, 1983, Proc. Natl. Acad. Sci. USA, *80*, 5383-5386). On obtient un adénovirus défectif pour les fonctions E1 et E4 (ΔE1, ΔE4) par transfection dans une lignée de complémentation pour E1 et E4 (par exemple la lignée de l'exemple 8) du mélange de ligation entre le génome de l'Ad dl324 et le plasmide pTG8500 ou pTG6588 linéarisé par *Spe*I.

D'autre part, on peut également procéder de la manière suivante. On clone le fragment *Spe*I-*Sca*I isolé de pTG1659 (exemple 2.3) dans le vecteur pTG6588 clivé par ces mêmes enzymes, pour obtenir pTG6591. Ce dernier comporte les séquences de l'Ad5 des nucléotides 21062 à 35935 mais, comme précédemment, délétées de l'intégralité de la région E4 et du fragment *Xba*I de la région E3. On introduit dans le vecteur pTG6591 digéré par *Pac*I, le fragment d'ADN synthétique décrit ci-dessus et on génère pTG6597. Les particules adénovirales peuvent être obtenues par recombinaison homologue entre l'ADN génomique de l'Ad dl324 clivé par *Spe*I et les plasmides pTG6591 ou pTG6597 clivé par *Bam*HI.

EXEMPLE 5 : Construction d'un virus "minimum"

Un vecteur adénoviral dit "minimum" est constitué par clonage dans un plasmide des éléments suivants :

- l'ITR 5' de l'Ad5 (des nucléotides 1 à 103) ;
- la région d'encapsidation de l'Ad5 (des nucléotides 104 à 458) ;
- une séquence nucléotidique exogène comprenant :
  * un premier gène d'intérêt thérapeutique placé de préférence sous le contrôle de son propre promoteur afin d'obtenir une régulation de l'expression la plus proche possible de la régulation naturelle,
  * un deuxième gène d'intérêt constitué du gène TK-HSV-1, et
  * de manière facultative, des séquences nucléotidiques quelconques ajoutées pour des raisons d'efficacité de replication ou d'encapsidation de manière à ce que la taille totale du génome à encapsider soit comprise entre 30 et 36kb ;
  * les séquences codant pour la protéine Gal4 de *Saccharomyces cerevisiae* (Laughon et Gesteland, 1984, Mol. Cell. Biol., *4*, 260-267) placées sous le contrôle d'un promoteur fonctionnel dans une cellule eucaryote supérieure ; et
- l'ITR 3' de l'Ad5 (des nucléotides 35833 à 35935).

L'assemblage de ces différents éléments est réalisé selon les techniques standards de biologie moléculaire. L'obtention de virions infectieux comprenant un tel vecteur se fait comme décrit précédemment dans une lignée de complémentation de l'exemple 7.

EXEMPLE 6 : Constitution d'une cellule de complémentation capable de complémenter *en trans* la fonction E1.

1. Constitution d'une cellule de complémentation comprenant la région E1 des nucléotides 100 à 5297 (pTG6533)

Celle-ci comporte :

- une cassette d'expression du gène pac, lequel est placé sous le contrôle du promoteur précoce du virus SV40 (nucléotides 5171 à 5243) et comprend en 3' le signal de terminaison de la transcription de SV40 (nucléotides 2543 à 2618). Le gène pac utilisé correspond à un fragment allant du nucléotide 252 au nucléotide 905 de la séquence divulguée par Lacalle et al. (1989, Gene, *79*, 375-380) et comportant 4 mutations par rapport à la séquence publiée (C en position 305 remplacé par A ; C en position 367 remplacé par T ; insertion d'un G en position 804 ; délétion d'un G en position 820),

- un fragment du génome de l'Ad5 allant des nucléotides 100 à 5297. Ce fragment comprend les régions E1A et E1B munies de leur propre promoteur et de leur signal de terminaison de la transcription ainsi qu'une fraction de la région E2, recouvrant ainsi les séquences codant pour la protéine IX - A titre indicatif, il semble que la lignée 293 ne soit pas capables de produire une protéine IX fonctionnelle.

La construction est réalisée en plusieurs étapes détaillées ci-après. Le vecteur p polyIII-I* (Lathe et al., 1987, Gene, *57*, 193-201) est soumis à une digestion par les enzymes *Acc*I et *Eco*RI. On clone dans le vecteur ainsi traité le fragment *Eco*RI-*Cla*I isolé du plasmide pTG6164. On obtient le vecteur pTG6528.

Le plasmide pTG6164 est issu de pLXSN (Miller D, 1989, Bio/Techniques, *7*, 980) et comprend le gène pac placé sous le contrôle du promoteur précoce du virus SV40. Brièvement, le fragment *Hind*III-*Kpn*I de pLXSN est introduit dans M13TG131 pour produire M13TG4194. On insère dans ce dernier, digéré par *Nhe*I et *Kpn*I, le fragment *Nhe*I-*Kpn*I de pMPSV H2 K IL2R (Takeda et al., 1988, Growth Factors, *1*, 59-66) pour produire M13TG4196. Celui-ci est digéré par *Hind*III-*Kpn*I et on clone le fragment issu d'une digestion *Hind*III et d'une digestion partielle *Kpn*I et purifié de pLXSN. On obtient pTG5192. Ce dernier est digéré par *Hind*III et partiellement par *Nhe*I et on introduit le fragment *Hind*III-*Nhe*I de pBabe Puro (Land et al., 1990, Nucleic Acids Res., *18*, 3587), donnant lieu à pTG6164.

Le vecteur pTG6528 est digéré par *Pst*I et on introduit au niveau de ce site le fragment *Pst*I isolé de pTG6185 (exemple 2.1) comportant le signal de terminaison de la transcription de SV40. On obtient pTG6529. Ce dernier est soumis à une digestion *Eco*RI-*Hpa*I et ligué à deux fragments, d'une part un fragment *Bsp*EI-*Bcg*I (positions 826 à 5297) purifié de l'ADN génomique d'Ad5 et d'autre part un fragment généré par PCR aux extrémités *Eco*RI et *Bsp*EI, pour donner pTG6531. Le fragment PCR est

généré par amplification génique à partir de l'ADN génomique d'Ad5 et des amorces OTG4564 et OTG4565 (reporté dans les SEQ ID NO: 27 et 28). Le fragment amplifié est digéré par les enzymes *Eco*RI et *Bsp*EI et mis en ligation comme indiqué dans le paragraphe précédent.

Le vecteur pTG6531 comprend les 2 unités de transcription (celle de la région E1 et celle du gène pac) dans la même orientation. Pour éviter des interférences au niveau de la transcription, on les place dans une orientation tête bêche (réverse l'une par rapport à l'autre) en traitant le pTG6531 par *Bam*HI et en religant. Le vecteur pTG6533 correspond à un clone présentant l'orientation reverse des deux unités.

Le vecteur pTG6533 est transfecté dans une lignée cellulaire mammifère, par exemple la lignée Vero (ATCC, CCL81) ou A549 (ATCC, CCL185) par la technique au phosphate de calcium. Les cellules transfectées sont cultivées selon les recommandations du fournisseur et sont placées 24 heures après la transfection en milieu sélectif contenant de la puromycine (concentration 6 µg/ml). On sélectionne les clones résistants sur lesquels on évalue l'expression des gènes de la région E1 afin de déterminer le clone le plus producteur, qui pourra être utilisé à titre de lignée de complémentation pour la préparation d'un adénovirus défectif pour la fonction E1, tel que celui détaillé dans l'exemple 2.

On analyse l'expression des séquences codant pour les protéines précoces de la région E1 par Northern blot en utilisant des sondes appropriées marquées à l'isotope ^{32}P. La production des protéines codées par la région E1A est détectée par immunoprécipitation après marquage des cellules à l'isotope ^{35}S et à l'aide d'un anticorps commercial (Oncogène Science Inc., référence DP11).

On peut également vérifier la capacité des produits d'expression de la région E1A à activer le promoteur de la région E1B (par analyse des ARNm E1B par Northern blot) ou à activer le promoteur de la région E2 (par dosage de l'activité enzymatique après transfection transitoire d'un plasmide "reporteur" comprenant le gène CAT (Chloramphénicol Acétyl Transférase) placé sous le contrôle du promoteur E2).

Enfin, on peut infecter ces cellules par l'Ad-RSV-βgal (Stratford-Perricaudet et al., 1992, *supra*) et titrer le virus par la technique agar dès qu'on observe un effet cytopathique. En général, on procède de la façon suivante : les cellules sont infectées à une moi (multiplicité d'infection) de 10. Environ 48 heures après l'infection, l'effet cytopathique étant visible, on lyse les cellules et on dose l'activité β-galactosidase selon le protocole

conventionnel (voir par exemple Maniatis et al., 1989, *supra*). Les clones positifs sont réinfectés à une moi plus faible. 48 heures après l'infection, on récolte le surnageant et les cellules selon les techniques classiques. On détermine le titre viral par la méthode sous agar en utilisant des cellules 293. Le rapport du titre obtenu sur le titre de départ constitue le facteur d'amplification.

2. *Construction d'une lignée de complémentation comprenant la région E1 des nucléotides 505 à 4034 (pTG6557, pTG6558, pTG6559, pTG6564 et pTG6565)*

Les vecteurs pTG6557, pTG6558 et pTG6559 comprennent :

(i) une cassette d'expression du gène pac (nucléotides 252 à 905 comme précédemment) sous le contrôle :

- du promoteur E2A de l'Ad2 (nucléotides 27341 à 27030) (dans pTG6558),

- du promoteur E2A de l'Ad2 délété des séquences comprises entre les nucléotides 27163 à 27182 (pour pTG6557). Une telle mutation permet de diminuer le niveau de base du promoteur E2A, sans affecter l'inductibilité par la protéine trans-activatrice codée par E1A, ou

- du promoteur précoce SV40 pour pTG6559.

Dans les trois cas, elle comporte également en 3' le signal de terminaison de la transcription du virus SV40 (nucléotides 2543 à 2618) ; et

(ii) une cassette d'expression comportant la partie de la région E1 de l'Ad5 allant des nucléotides 505 à 4034. Cette portion du génome adénoviral contient l'intégralité des séquences codant pour les protéines précoces de la région E1A, le signal de terminaison de la transcription de l'unité E1A, le promoteur E1B (inductible par la protéine trans-activatrice codée par E1A) et l'intégralité des séquences codantes de la région E1B. Elle inclut également les séquences codant pour la protéine IX, qui chevauchent la région E1B. Cependant, elle est dépourvue du promoteur de la région E1A et du signal de terminaison de la transcription des unités transcriptionnelles E1B et IX. Afin de permettre l'expression des séquences de la région E1, on introduit en 5' du fragment adénoviral, le promoteur du gène murin PGK et en 3' le signal de terminaison de la transcription du gène ß-globine de lapin

(nucléotides 1542 à 2064 de la séquence divulguée dans la banque de donnée Genebank sous la référence K03256).

De manière facultative, on peut également introduire des séquences nucléotidiques quelconques, par exemple isolées de pBR322 (Bolivar et al., 1977, Gène, *2*, 95-113), entre les cassettes d'expression du gène pac et de la région E1 ; afin d'éviter d'éventuelles interférences transcriptionnelles.

La construction de ces vecteurs s'effectue en plusieurs étapes reportées ci-dessous.

En premier lieu, on amplifie par PCR, la partie du génome de l'Ad5 allant du nucléotide 505 au nucléotide 826 à partir d'une préparation génomique et des amorces OTG5013 qui comprend en 5' un site *Pst*I utile pour les étapes de clonage ultérieures (SEQ ID NO: 29) et OTG4565 chevauchant le site *Bsp*E1 (SEQ ID NO: 28). Le fragment généré par PCR, est traité à l'ADN polymérase Klenow puis introduit dans le site *Sma*I de M13mp18 donnant lieu à M13TG6512. La séquence du fragment PCR est vérifiée.

Le vecteur pTG6533 (exemple 6.1) est digéré par les enzymes *Eco*RI et *Bsp*E1. On ligue le vecteur ainsi traité avec, d'une part, le fragment *Pst*I-*Bsp*E1 isolé de M13TG6512 et, d'autre part, le fragment *Eco*RI-*Pst*I isolé de pKJ-1. Ce dernier comprend la portion du promoteur du gène murin PGK, située entre les nucléotides -524 et -19, dont la séquence est reportée dans Adra et al. (1987, Gene, *60*, 65-74),. Cette étape donne lieu au pTG6552 et permet d'insérer le promoteur du gène murin PGK en amont de la région E1 de l'Ad5 débutant au nucléotide 505.

Par ailleurs, le fragment *Xho*I-*Bam*HI, dont l'extrémité générée par *Xho*I est rendue franche suite au traitement par l'ADN polymérase Klenow, est purifié de pBCMG Neo (Karasuyama et al., 1989, J. Exp. Med., *169*, 13-25). Ce fragment, qui comprend le signal de terminaison de la transcription du gène ß-globine de lapin, est introduit entre les sites *Sma*I et *Bam*HI du vecteur p polyII-Sfi/Not-14* (Lathe et al., 1987, Gene, *57*, 193-201). Le vecteur pTG6551 qui résulte, est quant à lui digéré par les enzymes *Sph*I et *Eco*RV afin d'y insérer un fragment du génome de l'Ad5 allant du nucléotide 3665 au nucléotide 4034. Ce fragment est généré par PCR selon le protocole standard. On utilise une préparation d'ADN génomique d'Ad5 à titre de matrice et les amorces OTG5015 qui recouvre le site interne *Sph*I en position 3665 (SEQ ID NO: 30) et OTG 5014 comprenant en 5' un site *Bgl*II (SEQ ID NO: 31).

Le fragment PCR est traité par l'ADN polymérase Klenow avant d'être cloné dans le site *Sma*I de M13mp18, générant M13TG6516. Après vérification de sa séquence, le fragment PCR est resorti par digestion par *Bgl*II, traitement à l'ADN polymérase Klenow et digestion par *Sph*I. Il est inséré entre les sites *Sph*I et *Eco*RV de pTG6551. Il en résulte pTG6554.

D'autre part, le vecteur pTG6529 (exemple 6.1) est soumis à une digestion par les enzymes *Hpa*I et *Hind*III. On purifie le fragment de 2,9 kb comportant le gène pac suivi du signal de terminaison de la transcription du virus SV40. Celui-ci est ligué au fragment *Sma*I-*Hind*III isolé de pE2 Lac (Boeuf et al., 1990, Oncogene, *5*, 691-699) qui porte le promoteur E2A de l'Ad2. On obtient le vecteur pTG6556. De manière alternative, il peut être ligué au fragment *Sma*I-*Hind*III isolé de pE2 Lac D9170 (Zajchowski et al., 1985, EMBO J., *4*, 1293-1300) qui porte le promoteur E2A muté de l'Ad2. On obtient, dans ce cas, pTG6550.

pTG6556 est digéré par les enzymes *Eco*RI et *Bam*HI. On insère entre ces sites, le fragment *Eco*RI-*Sac*II isolé de pTG6552 et le fragment *Sac*II-*Bam*HI isolé de pTG6554. On obtient le vecteur pTG6558. La même étape réalisée sur pTG6550 et pTG1643 (exemple 7.1) génère pTG6557 et pTG6559 respectivement.

pTG6557 et pTG6558 sont digérés par *Eco*RV, site unique situé entre les deux cassettes d'expression (gène pac et région E1). On clone dans ce site, un fragment *Eco*RV-*Pvu*II de 1,88kb isolé de pBR322 (Bolivar et al., *supra*), afin d'éloigner les deux promoteurs. On génère respectivement pTG6564 et pTG6565.

Les vecteurs pTG6557, pTG6558, pTG6559, pTG6564 et pTG6565 sont transfectés dans la lignée cellulaire A549. Comme précédemment, on sélectionne les clones résistant à la puromycine et on vérifie l'expression de la région E1. Les clones exprimant E1 sont destinés à amplifier et propager des adénovirus défectifs pour la fonction E1. La production des produits d'expression de E1 s'accompagne d'un effet cytotoxique mais l'analyse par Southern ne permet pas de mettre en évidence des réarrangements de vecteurs. Après infection par l'Ad-RSV - βgal, plusieurs clones sont capables d'amplifier le virus d'un facteur supérieur à 100.

3. *Construction d'une cellule de complémentation inductible par la protéine Gal4 de Saccharomyces cerevisiae.*

Ces vecteurs comprennent comme précédemment la partie de la région E1 de l'Ad5 allant du nucléotide 505 à 4034. Cependant l'expression des séquences de la région E1A est placée sous le contrôle d'un promoteur inductible constituée d'une part du promoteur minimal MLP d'Ad2 (TATA box et signal d'initiation de la transcription ; nucléotides -34 à +33) et d'autre part d'une séquence d'activation du gène Gal 10 activable par la protéine Gal4. La séquence consensus d'activation de 17 nucléotides (17MX), qui correspond au site de fixation de Gal4 est spécifiée dans Webster et al. (1988, Cell, *52*, 169). Le signal de terminaison de la transcription du gène de la β-globine de lapin est placé en 3' de l'unité transcriptionnelle E1B.

On synthétise un premier fragment d'ADN comprenant un dimère de la séquence 17MX (SEQ ID NO: 32 et 33) suivi du promoteur minimal MLP d'Ad2 et muni en son extrémité 5' d'un site *Sal*I et en son extrémité 3' d'un site *Bam*HI. Le site *Sal*I est rendu franc par traitement à l'ADN polymérase klenow. Par ailleurs, on synthétise un second fragment d'ADN comprenant un pentamère de la séquence suivi du même promoteur et muni en 5' et 3' des sites *Xba*I et *Bam*HI. Après digestion par *Xba*I, l'extrémité est rendue franche par traitement à la Klenow polymérase.

Chacun de ces fragment est introduit dans le site *Bgl*II de p poly II pour générer respectivement pTG1656 et pTG1657. Puis on introduit dans chacun des vecteurs préalablement digérés par *Pst*I-*Bam*HI, les deux fragments suivants : le fragment *Pst*I-*Xba*I isolé de pTG6552 (Exemple 6.2) et le fragment *Xba*I-*Bam*HI isolé de pTG6559 (exemple 6.2). On obtient pTG1660 et pTG1661 respectivement (Figure 5).

Les cellules A549 sont co-transfectées avec pTG1643 (vecteur d'expression du gène pac) et soit pTG1660 soit pTG1661. Les clones sont sélectionnés pour leur résistance à la puromycine et étudiés comme indiqué précédemment. Environ 50% des clones A549-1660 et A549-1661 produisent des produits d'expression de la région E1. Cependant, la production s'accompagne d'un effet cytotoxique, modifiant l'aspect morphologique des cellules.

L'intégration et le non réarrangement des plasmides dans le génome cellulaire est vérifié par Southern. Aucune modification substantielle des plasmides intégrés (pTG1643, pTG1660 et pTG1661) ne peut être mise en évidence dans les clones producteurs analysés. On peut également vérifier l'inductibilité de l'expression des séquences codées par la région E1A en présence de Gal4 (par transformation par un plasmide permettant l'expression constitutive de la protéine Gal4).

Après l'infection de plusieurs clones producteurs par l'Ad-RSV-Bgal à une moi d'environ 2, deux clones A549-1660 sont capables d'amplifier le stock viral d'un facteur supérieur à 100.

EXEMPLE 7 : Constitution d'une lignée de complémentation pour l'ensemble des fonctions essentielles à la réplication d'un adénovirus.

On construit un vecteur comprenant l'ensemble du génome adénoviral de l'Ad5 à l'exception de l'ITR 5', l'ITR 3' et la région d'encapsidation.

Le vecteur pTG6528 (exemple 6.1) est digéré par les enzymes *Pst*I et *Bgl*II entre lesquels on insère un fragment d'ADN synthétisé chimiquement selon le protocole standard constitué des oligonucléotides des OTG5039 et OTG5040 (SEQ ID NO: 34 et 35). La séquence des oligonucléotides est conçue de manière à ne pas reconstituer le site de clonage *Pst*I et introduire un site *Eco*RV. On obtient pTG1639, lequel est linéarisé par digestion par *Eco*RV et ligué à un fragment *Xba*I-*Bam*HI dont les extrémités sont rendues franches par traitement à l'ADN polymérase Klenow. Ce fragment est porteur du signal de terminaison de la transcription du virus SV40. Tout plasmide comportant un signal entouré des sites de restriction adéquates peut être utilisé à cette étape.

Le vecteur pTG1640 ainsi généré, est digéré par *Bam*HI et *Bgl*II et le fragment porteur de la cassette d'expression du gène pac est introduit dans le site *Bgl*II du vecteur pPolyII-Sfi/Not-14*. On obtient le pTG1641. Celui-ci est linéarisé par *Not*I et traité à l'ADN polymérase Klenow. On introduit le fragment *Bam*HI-*Sal*I de 0,276 kb isolé de pBR322 (Bolivar et al., *supra*) également traité à l'ADN polymérase Klenow. Ceci donne lieu à pTG1643.

Le pTG1643 est linéarisé par *Xho*I et on insère dans ce site un fragment hybride *Xho*I comportant un dimère 17MX suivi du promoteur minimum du gène TK-HSV-1 (nucléotides 303 à 450 de la séquence divulguée dans la banque de donnée Genebank sous la référence V00467 et complétée en 3' d'un site *Xho*I). On obtient le pTG1647 dans lequel le promoteur hybride 2x17MX-TK-HSV-1 est inséré dans la même orientation que la cassette d'expression du gène pac.

Cette construction, pTG1647, sert de vecteur de base pour introduire entre les sites *Pst*I et *Bam*HI un fragment du génome de l'Ad5 allant du nucléotide 505 au nucléotide 35826. Dans un premier temps, le pTG1647 est digéré par *Pst*I et *Bam*HI puis ligué, d'une part, au fragment *Pst*I-*Cla*I de pTG6552 (exemple 6.2) comportant la partie du

génome de l'Ad5 des nucléotides 505 à 918 et, d'autre part, au fragment *Cla*I-*Bam*HI (positions 918 à 21562) préparé à partir de l'ADN génomique de l'Ad5. Le vecteur ainsi obtenu, comporte la partie 5' de l'Ad5 à l'exception de l'ITR5' et de la région d'encapsidation.

Par ailleurs, la partie 3' du génome de l'Ad5 est assemblée dans le vecteur ppolyII-Sfi/Not-14*. Ce dernier est linéarisé par *Bam*HI et on introduit le fragment *Bam*HI-*Avr*II (nucléotides 21562 à 28752) du génome de l'Ad5 et un fragment PCR correspondant aux nucléotides 35463 à 35826 de l'Ad5. Ce dernier est généré à partir de l'ADN génomique de l'Ad5 et des amorces OTG5024 (SEQ ID NO: 36) et OTG5025 (SEQ ID NO: 37) et comporte en 5' un site *Bam*HI. Le vecteur obtenu est digéré par *Avr*II et on insère le fragment *Avr*II isolé de l'ADN génomique de l'Ad5 et s'étendant des positions 28753 à 35462.

Le fragment *Bam*HI comportant les séquences adénovirales est introduit dans le site *Bam*HI du vecteur de l'étape précédente comportant la partie 5' du génome adénoviral dépourvu de l'ITR 5' et la région d'encapsidation.

Une lignée de complémentation capable de complémenter l'ensemble des fonctions d'un adénovirus défectif est générée par transfection dans une lignée cellulaire, comme A549, selon le protocole décrit dans les exemples précédents.

On peut également procéder en construisant quatre vecteurs comportant la quasi totalité du génome adénoviral qui sera réassemblé sur un seul vecteur lors de l'étape finale.

- pTG1665 correspond au clonage du fragment *Bsp*E1 (nucléotides 826 à 7269) isolé d'une préparation d'ADN génomique d'Ad5, dans le site *Xma*I de p poly II-Sfi/Not-14 * ;

- pTG1664 est généré par l'insertion du fragment *Not*I (nucléotides 6503 à 1504) isolé d'une préparation d'ADN génomique d'Ad5, dans le site *Not*I du même vecteur.

- pTG1662 est obtenu par introduction du fragment *Aat*II (nucléotides 10754 à 23970) isolé d'une préparation d'ADN génomique d'Ad5 dans le site *Aat*II de p polyII.

- pTG1659 comportant la partie 3' du génome d'Ad5 (exemple 2.3).

Puis on introduit un fragment comportant un système d'expression inductible comme le promoteur décrit à l'exemple 6.3 ou 7 inductible par Gal4 ou un promoteur de l'art antérieur comme le promoteur metallothionéine ou tétracycline. Un tel fragment est placé en amont des séquences 5' de l'Ad5 (nucléotides 505 à 918) dans le vecteur pTG1665 digéré par *Aat*II et *Cla*I. Enfin, on clone successivement dans le vecteur précédent et aux sites correspondants les fragments *Not*I de pTG1664, *Aat*II de pTG1662 et enfin *Bam*HI de pTG1659.

Une lignée de complémentation est générée par co-transfection du vecteur précédent et de pTG1643 et on isole les clones résistants à la puromycine. Cette lignée est plus particulièrement destinée à amplifier et encapsider les vecteurs adénoviraux de l'exemple 5 défectifs pour les fonctions E1, E2 et E4 et les fonctions tardives.

EXEMPLE 8 : Constitution d'une lignée de complémentation pour les fonctions E1 et E4.

Le vecteur pTG1647 (exemple 7) est digéré par les enzymes *Pst*I-*Bam*HI et on introduit dans le vecteur ainsi traité 3 fragments :

- le fragment *Pst*I-*Xba*I de pTG6552 (exemple 6.2) portant les séquences d'Ad5 du nucléotide 505 au nucléotide 1339,

- le fragment *Xba*I-*Sph*I de pTG6552 portant les séquences d'Ad5 du nucléotide 1340 au nucléotide 3665, et

- le fragment *Sph*I-*Bam*HI de pTG6554 (exemple 6.2) portant les séquences d'Ad5 du nucléotide 3665 à 4034 et un signal de terminaison de la transcription.

Le vecteur ainsi obtenu est coupé par *Bam*HI et on introduit dans ce site trois fragments qui sont les suivants :

- un fragment digéré par *Bam*HI-*Afl*II généré par PCR correspondant à la séquence de l'Ad5 situé entre les positions 32800 à 33104. On utilise l'ADN génomique d'Ad5 comme matrice et les amorces OTG5078 (SEQ ID NO: 38) et OTG5079 (SEQ ID NO: 39),

- le fragment *Afl*II-*Avr*II isolé de l'ADN génomique d'Ad5 (nucléotides 33105 à 35463),

- le fragment *Avr*II-*Bam*HI généré par PCR à l'aide des amorces OTG5024 et OTG5025 (voir exemple 7).

Le vecteur ainsi généré est introduit dans une lignée cellulaire selon le protocole décrit précédemment, pour constituer une lignée de complémentation pour les fonctions E1 et E4.

Par ailleurs, une telle lignée peut également être obtenue selon le protocole suivant :

La région E4 du génome de l'Ad5 (nucléotides 32800 à 35826) est reconstituée en plusieurs étapes. La partie allant des nucléotides 33116 à 32800 est synthétisée par PCR à partir de l'ADN génomique d'Ad5 avec le couple d'amorces OTG5078 et OTG5079 (SEQ ID NO: 38 et 39), puis insérée dans le site *Eco*RV de M13TG130, pour générer M13TG1645.

Le fragment *Bam*HI-*Afl*II de ce dernier est engagé dans une réaction de ligation avec le fragment *Afl*II-AvrII d'Ad5 (nucléotides 33104 à 35463) et le vecteur pTG7457 digéré par *Bam*HI et *Avr*II. On obtient pTG1650.

Puis on complète la région E4 par obtention du fragment correspondant aux nucléotides 35826 à 35457 par PCR à partir d'une préparation d'ADN génomique d'Ad5 et des amorces OTG5024 et OTG5025 (SEQ ID NO: 36 et 37). Celui-ci est inséré dans le site *Sma*I de M13mp18 pour donner M13TG1646. Le fragment *Avr*II-*Eco*RI est isolé de ce dernier et cloné entre les sites *Avr*II et *Eco*RI de pTG1650. On obtient pTG1652.

Le fragment *Bam*HI comportant la région E4 d'Ad5 est isolé de pTG1652 et cloné dans le site *Bam*HI de pTG1643, de pTG6559 (exemple 6.2) ou dans le site *Ssp*I de pTG6564 (exemple 6.2) après avoir rendu les sites francs, pour générer pTG1653, pTG1654 et pTG1655 (Figure 6) respectivement.

On génère par des techniques conventionnelles une cellule de complémentation capable de complémenter *en trans* les fonctions E1 et E4, par :

(1) transformation de pTG1653 dans la lignée cellulaire 293, ou
(2) transformation de pTG1654 ou pTG1655 dans la lignée cellulaire A549.

D'une manière générale, l'expression des produits des régions E1 et E4 s'accompagne d'un effet cytotoxique. Un certain nombre de clones 293-1653 est capable de complémenter à la fois des adénovirus délétés de E1 et des adénovirus délétés de E4.

Une autre alternative consiste à procéder de la manière suivante.

Le vecteur M13TG1646 est soumis à une mutagénèse dirigée avec l'oligonucléotide mutagène OTG5991 (SEQ ID NO: 40) dans le but de déléter le promoteur de la région E4 et d'insérer un site *Hpa*I. Le vecteur muté est désigné M13TG6522. Il est digéré par *Pst*I, traité à l'ADN polymérase du phage T4 puis par *Avr*II et mis en ligation avec un fragment *Eco*RI (Klenow)-*Avr*II purifié de pTG1652 (exemple 8), pour donner pTG6595. Ce dernier est clivé par *Hpa*I et on introduit le fragment de 0,8 kb obtenu de pTG5913 (Figure 7) après digestion *Bgl*II et *Bam*HI et traitement à la Klenow. On génère pTG6596 dans lequel la région E4 (positions 32800 à 35826) est placée sous le contrôle du promoteur TK. A titre indicatif, pTG5913 porte le gène TK-HSV-1 et le fragment *Bgl*II-*Bam*HI correspond au promoteur de ce gène (Wagner et al., 1981, Proc. Natl. Acad. Sci., USA, *78*, 1441 - 1445).

Parallèlement, les vecteurs pTG1643 et pTG6559 (exemple 6) sont linéarisés par *Bam*HI et on insère un fragment synthétique issu de la réassociation des oligonucléotides OTG6141 et OTG6142 (SEQ ID NO: 41 et 42), pour obtenir respectivement pTG8508 et pTG8507. Ces derniers sont clivés par *Bam*HI avant d'introduire le fragment *Bam*HI purifié de pTG6596 comportant la cassette d'expression de E4. On génère les vecteurs pTG8512 (Figure 8) et pTG8513 (Figure 9).

D'autre part, l'introduction du fragment *Bam*HI de pTG1652 dans le vecteur pTG8508 ou pTG8507 linéarisé par la même enzyme aboutit à pTG8514 et pTG8515 respectivement (Figures 10 et 11).

Les lignées cellulaires transfectées par pTG8512 ou pTG8515 permettront de complémenter un adénovirus défectif pour la fonction E4, alors que celles résultant de la transfection de pTG8513 ou pTG8514 sont destinées à amplifier et propager des adénovirus défectifs pour les fonctions E1 et E4. De même, la transfection de pTG8512 ou pTG8515 dans les cellules 293 permettront de complémenter des adénovirus défectifs pour E1 et E4.

LISTE DE SEQUENCES

(1) INFORMATION GENERALE:

(i) DEPOSANT:
(A) NOM: TRANSGENE
(B) RUE: 11, rue de Molsheim
(C) VILLE: STRASBOURG
(E) PAYS: FRANCE
(F) CODE POSTAL: 67082
(G) TELEPHONE: (33) 88.27.91.00
(H) TELECOPIE: (33) 88.22.58.07

(ii) TITRE DE L' INVENTION: Nouveaux adenovirus defectifs et lignees de complementation correspondantes

(iii) NOMBRE DE SEQUENCES: 42

(iv) FORME LISIBLE PAR ORDINATEUR:
(A) TYPE DE SUPPORT: Tape
(B) ORDINATEUR: IBM PC compatible
(C) SYSTEME D' EXPLOITATION: PC-DOS/MS-DOS
(D) LOGICIEL: PatentIn Release #1.0, Version #1.25 (OEB)

(2) INFORMATION POUR LA SEQ ID NO: 1:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 30 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG4174)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 1:

GTGACGTCTT TGGTGTTTTC GCGGGAAAAC 30

(2) INFORMATION POUR LA SEQ ID NO: 2:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 30 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG4173)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 2:

ACCGAGTAAG ATTTGTCTAG GGCCGCGGGG 30

(2) INFORMATION POUR LA SEQ ID NO: 3:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 33 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG4191)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 3:

GGCCATGGTC GCGGGAAAGG GACTTTGACC GTT 33

(2) INFORMATION POUR LA SEQ ID NO: 4:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 31 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5021)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 4:

GAACGGATCC CCAGACTCTG TTTGGATTTG G 31

(2) INFORMATION POUR LA SEQ ID NO: 5:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 30 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5157)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 5:

CCAGAAATAT CTTCGCCCAG GCCGCCGCCC 30

(2) INFORMATION POUR LA SEQ ID NO: 6:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 20 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5564)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 6:

GATCCGATAT CCCGTTAACC 20

(2) INFORMATION POUR LA SEQ ID NO: 7:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 20 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5565)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 7:

GATCGGTTAA CGGGATATCG 20

(2) INFORMATION POUR LA SEQ ID NO: 8:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 47 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5892)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 8:

GTCGTAGGAT CCAGCTGCTC CCTGCTTGTG TGTTGGAGGT CGCTGAG 47

(2) INFORMATION POUR LA SEQ ID NO: 9:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 47 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5893)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 9:

GTAGCTGACG TCCCAGGTGC ACACCAATGT GGTGAATGGT CAAATGG 47

(2) INFORMATION POUR LA SEQ ID NO: 10:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 46 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5920)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 10:

ACGGTAGGAT CCGACGTCGG TGAGCTCCTC GCTTGGTCTC CGTCCG 46

(2) INFORMATION POUR LA SEQ ID NO: 11:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 24 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5891)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 11:

CAACCCCGAT TCTAGAGAAA CCTG 24

(2) INFORMATION POUR LA SEQ ID NO: 12:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 35 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6079)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 12:

GCGCAGTTGC TCTGCGGATC CACTTAACAT TCAGT 35

(2) INFORMATION POUR LA SEQ ID NO: 13:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 38 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6080)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 13:

TAAAAGTACC AGGTAAGGAT CCCCTTGGTT TGCTTGGG 38

(2) INFORMATION POUR LA SEQ ID NO: 14:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 21 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6064)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 14:

GAAACCGAAT TCTCTTGGAA C 21

(2) INFORMATION POUR LA SEQ ID NO: 15:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 32 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6065)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 15:

ACGAATGCAG CTCTCCACTT AACATTCAGT CG 32

(2) INFORMATION POUR LA SEQ ID NO: 16:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 27 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5481)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 16:

CAGTGAATTC ATCATCAATA ATATACC 27

(2) INFORMATION POUR LA SEQ ID NO: 17:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 24 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple

(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5482)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 17:

AAACTGGTCA CCGTGATTAA AAAG 24

(2) INFORMATION POUR LA SEQ ID NO: 18:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 25 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5455)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 18:

ATCGGAATTC AAGATGATTA GGTAC 25

(2) INFORMATION POUR LA SEQ ID NO: 19:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 28 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5456)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 19:

ATCGTCTAGA TTAAGGCATT TTCTTTTC 28

(2) INFORMATION POUR LA SEQ ID NO: 20:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 18 paires de bases

(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5728)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 20:

TGTAGCAGGA GGACTAAG 18

(2) INFORMATION POUR LA SEQ ID NO: 21:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 39 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5729)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 21:

CCGCATTAAT TAACCGCGAC AAACGATTCT TTATTCTTG 39

(2) INFORMATION POUR LA SEQ ID NO: 22:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 36 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (5730)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 22:

CGCGGTTAAT TAATGCGGTA AAACCTACGT CACCCG 36

(2) INFORMATION POUR LA SEQ ID NO: 23:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 30 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6060)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 23:

AATAAAAGAT CATTATTTTC ATTAGAACTG 30

(2) INFORMATION POUR LA SEQ ID NO: 24:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 24 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6061)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 24:

TGTGTTGGTT TTTTGTGTGT TAAT 24

(2) INFORMATION POUR LA SEQ ID NO: 25:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 30 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6062)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 25:

TAACACACAA AAAACCAACA CACAGTTCTA 30

(2) INFORMATION POUR LA SEQ ID NO: 26:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 24 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6063)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 26:

ATGAAAATAA TGATCTTTTA TTAT 24

(2) INFORMATION POUR LA SEQ ID NO: 27:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 32 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG4564)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 27:

TCCGTGAATT CTAGTAGTGT GGCGGAAGTG TG 32

(2) INFORMATION POUR LA SEQ ID NO: 28:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 23 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG4565)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 28:

TCCAGTCCGG AGAACCGGGC GCC 23

(2) INFORMATION POUR LA SEQ ID NO: 29:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 28 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5013)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 29:

TAACCTGCAG GAGTGCCAGC GAGTAGAG 28

(2) INFORMATION POUR LA SEQ ID NO: 30:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 21 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonuclotide de synthese (OTG5015)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 30:

CAACGCGCAT GCCCCCATGG G 21

(2) INFORMATION POUR LA SEQ ID NO: 31:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 31 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5014)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 31:

TAGGAGATCT GTTTTAAACC GCATTGGGAG G 31

(2) INFORMATION POUR LA SEQ ID NO: 32:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 34 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 32:

CGGAGTACTG TCCTCCGCGG AGTACTGTCC TCCG 34

(2) INFORMATION POUR LA SEQ ID NO: 33:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 34 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 33:

CGGAGGACAG TACTCCGCGG AGGACAGTAC TCCG 34

(2) INFORMATION POUR LA SEQ ID NO: 34:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 16 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5039)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 34:

TGCTGGATAT CAGTCA 16

(2) INFORMATION POUR LA SEQ ID NO: 35:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 24 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5040)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 35:

GATCTGACTG ATATCCAGCA TGCA 24

(2) INFORMATION POUR LA SEQ ID NO: 36:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 20 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5024)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 36:

CTCCTGCCTA GGCAAAATAG 20

(2) INFORMATION POUR LA SEQ ID NO: 37:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 32 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5025)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 37:

GCAGATGGAT CCGGGCGGAG TAACTTGTAT GT 32

(2) INFORMATION POUR LA SEQ ID NO: 38:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 31 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5078)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 38:

GTCGCGGATC CGTTATGTTT CAACGTGTTT A 31

(2) INFORMATION POUR LA SEQ ID NO: 39:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 20 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonuleotide de synthese (OTG5079)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 39:

ACATGAACTT AAGCGAGCTG 20

(2) INFORMATION POUR LA SEQ ID NO: 40:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 38 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG5991)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 40:

CACGGCACCA GCTCAAGTTA ACGGATCCAT CTGCGGGT 38

(2) INFORMATION POUR LA SEQ ID NO: 41:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 27 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: NON

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6141)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 41:

GATCCTGTGT GTTGGTTTTT TGTGTGC 27

(2) INFORMATION POUR LA SEQ ID NO: 42:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 27 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(iii) HYPOTHETIQUE: NON

(iii) ANTI-SENS: OUI

(vi) ORIGINE:
(A) ORGANISME: Oligonucleotide de synthese (OTG6142)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 42:

GATCGCACAC AAAAAACCAA CACACAG 27

Revendications

1. Un vecteur adénoviral défectif pour la réplication, capable d'être encapsidé dans une cellule de complémentation, qui dérive du génome d'un adénovirus comprenant de 5' en 3', un ITR 5', une région d'encapsidation, une région E1A, une région E1B, une région E2, une région E3, une région E4 et un ITR 3', par délétion :

   (i) de tout ou partie de la région E1A, et de l'intégralité de la partie de la région E1B codant pour les protéines précoces ; ou

   (ii) de tout ou partie de la région E1A et de tout ou partie d'au moins une région sélectionnée parmi les régions E2 et E4 ; ou

   (iii) de tout ou partie de la région E1A et d'une partie de la région d'encapsidation.

2. Un vecteur adénoviral selon la revendication 1, qui dérive du génome d'un adénovirus par délétion de tout ou partie de la région E1A et de l'intégralité de la partie de la région E1B codant pour les protéines précoces.

3. Un vecteur adénoviral selon la revendication 2, qui dérive en outre du génome d'un adénovirus par délétion de tout ou partie de la région E3.

4. Un vecteur adénoviral selon la revendication 2 ou 3, qui dérive en outre du génome d'un adénovirus par délétion de tout ou partie de la région E2.

5. Un vecteur adénoviral selon l'une des revendications 2 à 4, qui dérive en outre du génome d'un adénovirus par délétion de tout ou partie de la région E4.

6. Un vecteur adénoviral selon la revendication 1, qui dérive du génome d'un adénovirus par délétion de tout ou partie de la région E1A et de tout ou partie de la région E2.

7. Un vecteur adénoviral selon la revendication 1, qui dérive du génome d'un adénovirus par délétion de tout ou partie de la région E1A et de tout ou partie de la région E4.

8. Un vecteur adénoviral selon la revendication 6 ou 7, qui dérive en outre du génome d'un adénovirus par délétion de tout ou partie de la région E1B.

9. Un vecteur adénoviral selon l'une des revendications 6 à 8, qui dérive en outre du génome d'un adénovirus par délétion de tout ou partie de la région E3.

10. Un vecteur adénoviral selon la revendication 6, 8 ou 9, qui dérive en outre du génome d'un adénovirus par délétion de tout ou partie de la région E4.

11. Un vecteur adénoviral selon l'une des revendications 3 à 5, 9 ou 10, qui dérive du génome d'un adénovirus par délétion partielle de la région E3 dudit génome en maintenant la partie de ladite région E3 codant pour la protéine gp19kDa.

12. Un vecteur adénoviral selon la revendication 11, dans lequel la partie de la région E3 codant pour la protéine gp19kDa est placée sous le contrôle des éléments appropriés à l'expression de ladite protéine dans la cellule hôte.

13. Un vecteur adénoviral selon l'une des revendications 1 à 12, qui dérive du génome d'un adénovirus par délétion de tout ou partie de la région E1A et d'une partie de la région d'encapsidation.

14. Un vecteur adénoviral selon la revendication 13, qui dérive du génome d'un adénovirus humain de type 5 par délétion de la partie de la région d'encapsidation :

    (i) s'étendant du nucléotide 270 au nucléotide 346 ;

    (ii) s'étendant du nucléotide 184 au nucléotide 273 ; ou

    (iii) s'étendant du nucléotide 287 au nucléotide 358.

15. Un vecteur adénoviral selon l'une des revendications 1 à 14, qui dérive du génome d'un adénovirus sélectionné parmi les adénovirus canins aviaires et humains.

16. Un vecteur adénoviral selon la revendication 15, qui dérive du génome d'un adénovirus humain de type 5.

17. Un vecteur adénoviral selon la revendication 16, qui dérive du génome d'un adénovirus humain de type 5 par délétion de la partie de la région E1B s'étendant du nucléotide 1634 jusqu'au nucléotide 4047 au moins.

18. Un vecteur adénoviral selon la revendication 16 ou 17, qui dérive du génome d'un adénovirus humain de type 5 notamment par délétion de la partie de la région E3 s'étendant du nucléotide 27871 jusqu'au nucléotide 30748.

19. Un vecteur adénoviral selon l'une des revendications 16 à 18, qui dérive du génome d'un adénovirus humain de type 5 par délétion de la partie de la région E4 s'étendant du nucléotide 32800 au nucléotide 35826.

20. Un vecteur adénoviral selon l'une des revendications 1 à 19, qui dérive du génome d'un adénovirus par délétion d'au moins 18 % du génome dudit virus.

21. Un vecteur adénoviral selon la revendication 20, qui dérive du génome d'un adénovirus par délétion d'au moins 22 % du génome dudit virus.

22. Un vecteur adénoviral selon la revendication 21, qui dérive du génome d'un adénovirus par délétion d'au moins 40 % du génome dudit virus.

23. Un vecteur adénoviral selon la revendication 22, qui dérive du génome d'un adénovirus par délétion d'au moins 95 % du génome dudit virus.

24. Un vecteur adénoviral selon la revendication 23, qui dérive du génome d'un adénovirus par délétion de l'ensemble du génome dudit adénovirus à l'exclusion des ITRs 5' et 3' et de tout ou partie de la région d'encapsidation.

25. Un vecteur adénoviral selon la revendication 24, qui dérive du génome d'un adénovirus humain de type 5 par délétion de la partie du génome viral s'étendant des nucléotides 459 à 35832.

26. Un vecteur adénoviral selon l'une des revendications 1 à 25, qui comprend en outre une séquence nucléotidique exogène.

27. Un vecteur adénoviral selon la revendication 26, qui comprend en outre un gène d'intérêt placé sous le contrôle des éléments nécessaires à son expression.

28. Un vecteur adénoviral selon l'une des revendications 26 ou 27, qui comprend en outre un gène codant pour une protéine trans-activatrice de transcription non adénovirale ; ledit gène étant placé sous le contrôle des éléments nécessaires à l'expression de ladite protéine dans une cellule hôte.

29. Un vecteur adénoviral selon la revendication 28, comprenant le gène codant pour la protéine trans-activatrice de transcription Gal4 de *Saccharomyces cerevisiae*.

30. Une particule d'adénovirus comprenant un vecteur adénoviral selon l'une des revendications 1 à 29.

31. Une cellule hôte eucaryote comprenant un vecteur adénoviral selon l'une des revendications 1 à 29 ou une particule d'adénovirus selon la revendication 30.

32. Une lignée de complémentation comportant un élément de complémentation, comprenant notamment une partie de la région E1 du génome d'un adénovirus à l'exclusion de l'ITR 5' ; ledit élément de complémentation étant capable de complémenter *en trans* un vecteur adénoviral défectif et étant intégré dans le génome de ladite lignée de complémentation ou inséré dans un vecteur d'expression.

33. Une lignée de complémentation selon la revendication 32, comprenant notamment :

(i) tout ou partie de la région E1A du génome d'un adénovirus ; et

(ii) tout ou partie d'au moins une région dudit génome sélectionnée parmi les régions E1B, E2 et E4.

34. Une lignée de complémentation selon la revendication 32, comprenant notamment :

(i) tout ou partie de la région E1A du génome d'un adénovirus ; et

(ii) tout ou partie d'au moins deux des régions E1B, E2 et E4 dudit génome.

35. Une lignée de complémentation selon la revendication 32, comprenant notamment :

(i) tout ou partie de la région E1A du génome d'un adénovirus ; et

(ii) tout ou partie des régions E1B, E2 et E4 dudit génome.

36. Une lignée de complémentation selon l'une des revendications 33 à 35, comprenant notamment tout ou partie de la région E1A et l'intégralité de la région E1B du génome d'un adénovirus codant pour les protéines précoces.

37. Une lignée de complémentation selon l'une des revendications 32 à 36, comprenant notamment une partie du génome d'un adénovirus sélectionné parmi les adénovirus canins, aviaires et humains.

38. Une lignée de complémentation selon la revendication 37, comprenant notamment une partie du génome d'un adénovirus humain de type 5.

39. Une lignée de complémentation selon la revendication 38, comprenant notamment la partie du génome d'un adénovirus humain de type 5 :

(i) s'étendant du nucléotide 100 au nucléotide 5297 ;

(ii) s'étendant du nucléotide 100 au nucléotide 4034 ; ou

(iii) s'étendant du nucléotide 505 au nucléotide 4034.

40. Une lignée de complémentation selon la revendication 38 ou 39, comprenant notamment la partie de la région E4 du génome d'un adénovirus humain de type 5 s'étendant du nucléotide 32800 au nucléotide 35826.

41. Une lignée de complémentation selon la revendication 38, comprenant notamment la partie du génome d'un adénovirus humain de type 5 s'étendant du nucléotide 505 au nucléotide 35826.

42. Une lignée de complémentation selon l'une des revendications 32 à 41, comprenant une partie de la région E1A du génome d'un adénovirus dépourvue de son promoteur naturel ; ladite partie étant placée sous le contrôle d'un promoteur approprié.

43. Une lignée de complémentation selon la revendication 42, dans laquelle ladite partie de la région E1A est placée sous le contrôle d'un promoteur inductible par une protéine trans-activatrice de transcription non-adénovirale.

44. Une lignée de complémentation selon la revendication 43, dans laquelle ladite partie de la région E1A est placée sous le contrôle d'un promoteur inductible par une protéine trans-activatrice de transcription codée par un vecteur adénoviral selon la revendication 28 ou 29.

45. Une lignée de complémentation selon la revendication 43 ou 44, dans laquelle ladite partie de la région E1A est placée sous le contrôle d'un promoteur inductible par la protéine trans-activatrice de transcription Gal4 de *Saccharomyces cerevisiae*.

46. Une lignée de complémentation selon l'une des revendications 32 à 45, comprenant en outre un gène codant pour un marqueur de sélection.

47. Une lignée de complémentation selon la revendication 46, dans laquelle le gène de sélection code pour la puromycine acetyl-transférase.

48. Une lignée de complémentation selon la revendication 46 ou 47, dans laquelle le gène de sélection est placé sous le contrôle d'un promoteur inductible par une protéine trans-activatrice de transcription codée par la région E1A du génome d'un adénovirus sauvage, notamment sous le contrôle du promoteur de la région E2 dudit génome.

49. Une lignée de complémentation selon l'une des revendications 32 à 48, dérivée d'une lignée cellulaire acceptable d'un point de vue pharmaceutique.

50. Une lignée de complémentation selon la revendication 49, dérivée d'une lignée cellulaire sélectionnée parmi les lignées Vero, BHK, A549, MRC5, W138 et CHO.

51. Une lignée de complémentation selon l'une des revendications 32 à 48, dérivée d'une cellule de la rétine d'un embryon humain.

52. Un procédé de préparation d'une particule d'adénovirus selon la revendication 30, selon lequel :

(i) on introduit un vecteur adénoviral selon l'une des revendications 1 à 29 dans une lignée de complémentation capable de complémenter *en trans* ledit vecteur adénoviral pour obtenir une lignée de complémentation transfectée ;

(ii) on cultive ladite lignée de complémentation transfectée dans des conditions appropriées pour permettre la production de ladite particule d'adénovirus ; et

(iii) on récupère ladite particule d'adénovirus dans la culture cellulaire.

53. Un procédé selon la revendication 52, selon lequel on met en oeuvre une lignée de complémentation selon l'une des revendications 32 à 51.

54. Usage thérapeutique ou prophylactique d'un vecteur adénoviral selon l'une des revendications 1 à 29, d'une particule d'adénovirus selon la revendication 30 ou obtenue en mettant en oeuvre un procédé selon la revendication 52 ou 53, d'une cellule hôte eucaryote selon la revendication 31 ou d'une lignée de complémentation selon l'une des revendications 32 à 51.

55. Une composition pharmaceutique comprenant à titre d'agent thérapeutique ou prophylactique un vecteur adénoviral selon l'une des revendications 1 à 29, une particule d'adénovirus selon la revendication 30 ou obtenue en mettant en oeuvre un procédé selon la revendication 52 ou 53, une cellule eucaryote selon la revendication 31 ou une lignée de complémentation selon l'une des revendications 32 à 51, en association avec un support acceptable d'un point de vue pharmaceutique.

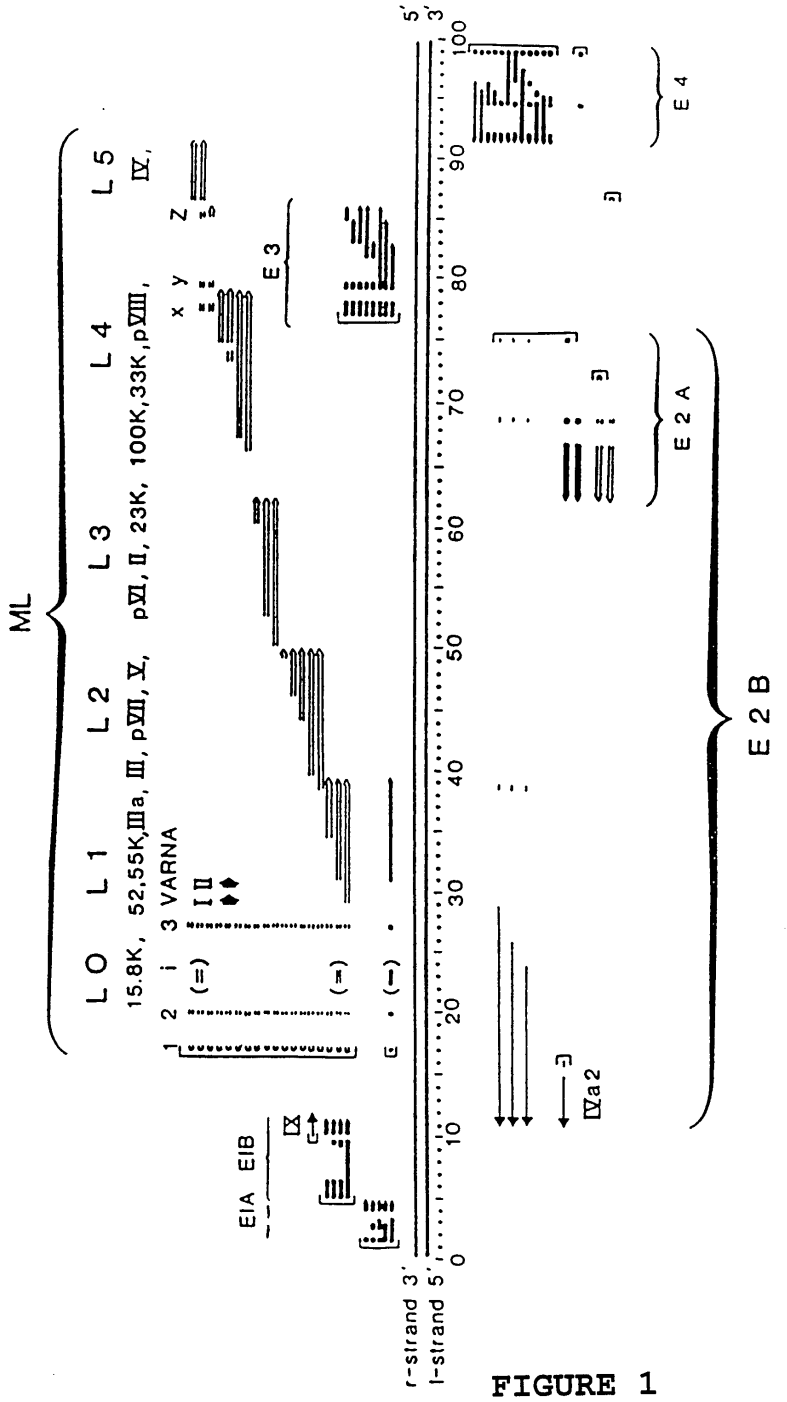
ML
L0 L1 L2 L3 L4 L5
15.8K, 52,55K,IIIa, III, pVII, V, pVI, II, 23K, 100K,33K,pVIII, IV,
1 2 i 3 VARNA
I II
x y Z
E3
E1A E1B
IX
IVa2
E2A
E2B
E4
0 10 20 30 40 50 60 70 80 90 100
5' 3'
r-strand 3'
l-strand 5'

FIGURE 1

pTG6546

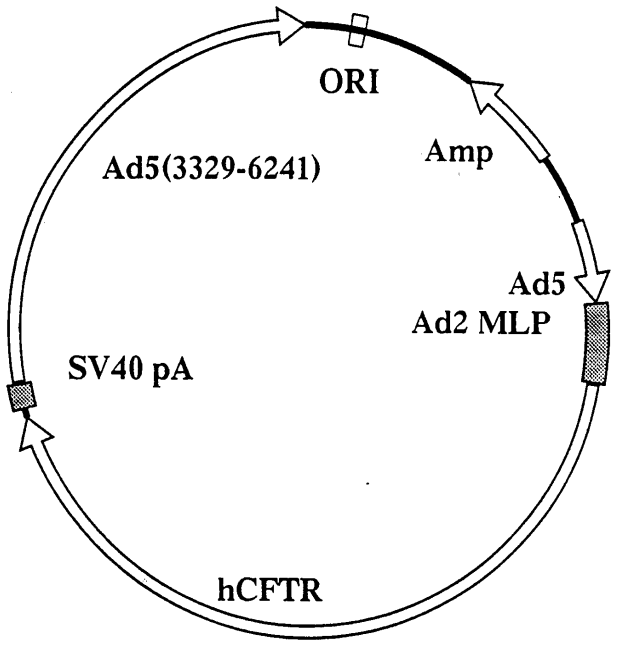

FIGURE 2

pTG6581

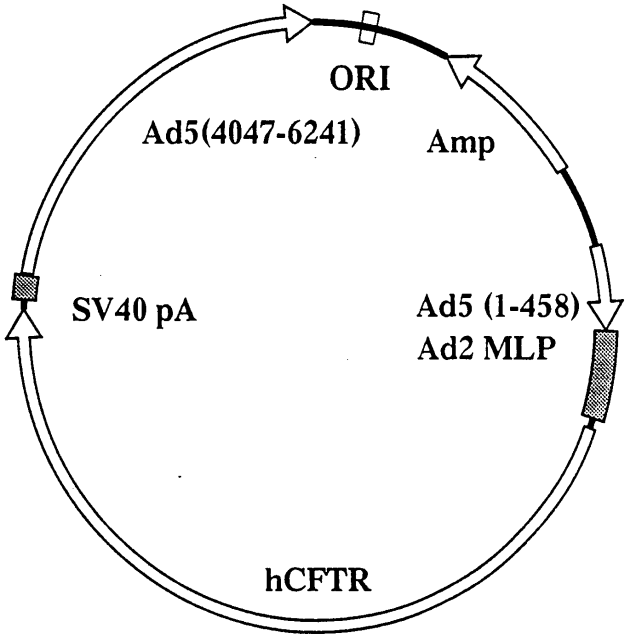

FIGURE 3

pTG6303

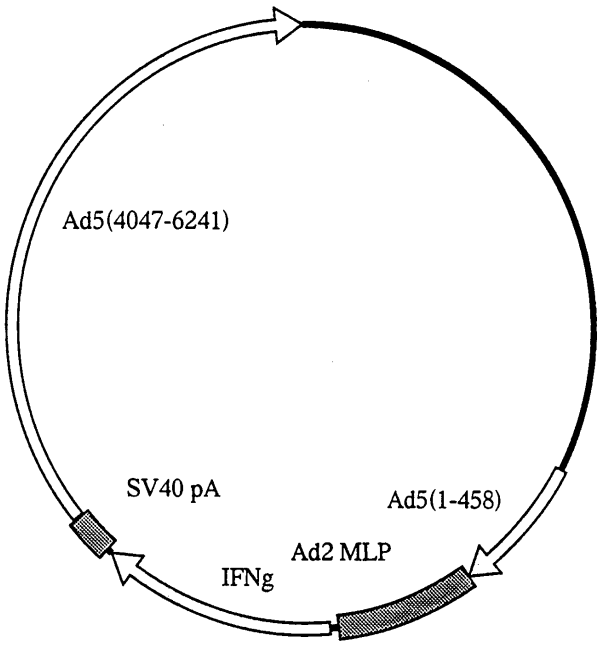

FIGURE 4

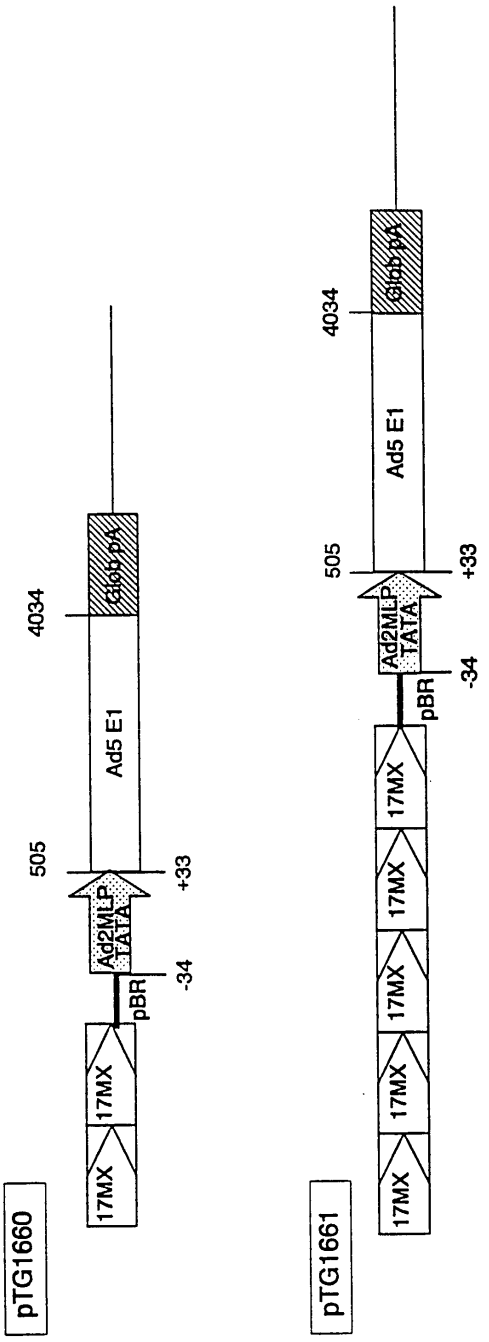
pTG1660
17MX
17MX
17MX
pBR
Ad2MLP TATA
-34
505
+33
Ad5 E1
4034
Glob pA
pTG1661
17MX
17MX
17MX
17MX
17MX
pBR
Ad2MLP TATA
-34
505
+33
Ad5 E1
4034
Glob pA

FIGURE 5

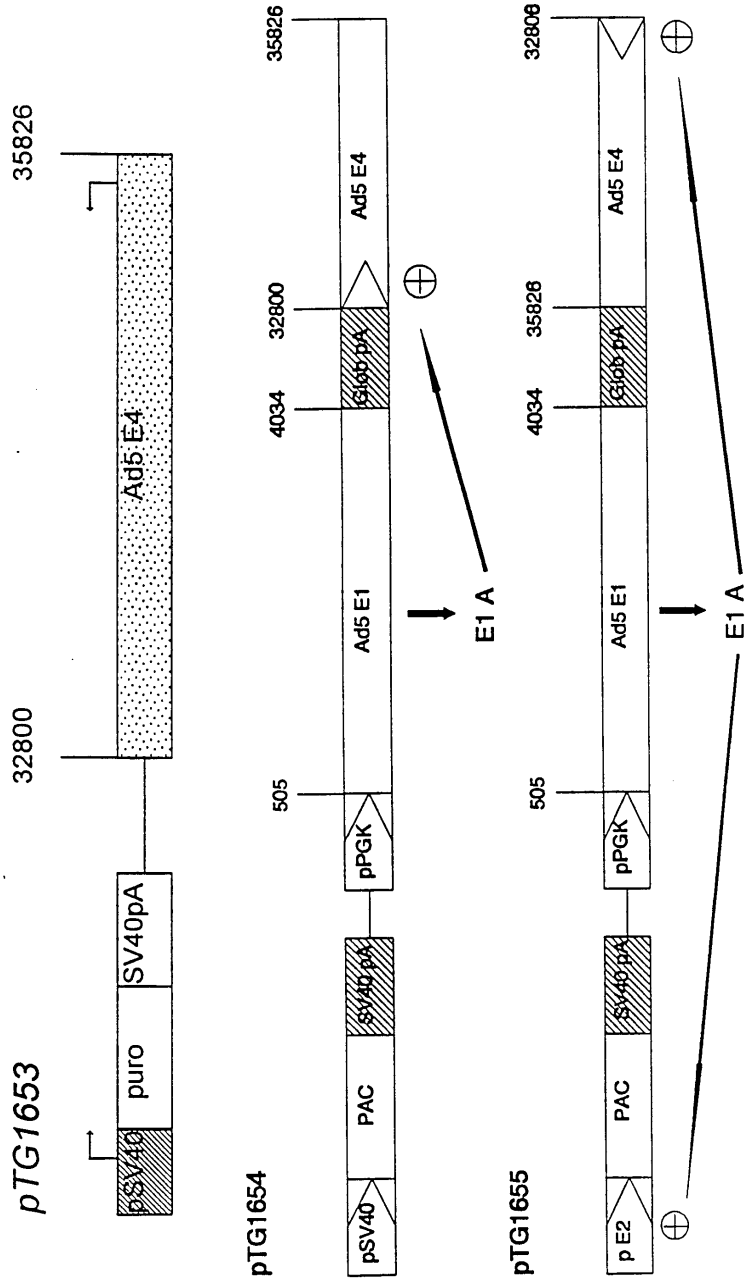
35826
32800
Ad5 E4
SV40pA
puro
pSV40
pTG1653
35826
32800
4034
505
Ad5 E4
Gl66 pA
Ad5 E1
pPGK
SV40 pA
PAC
pSV40
E1 A
pTG1654
32806
35826
4034
505
Ad5 E4
Gl66 pA
Ad5 E1
pPGK
SV40 pA
PAC
p E2
E1 A
pTG1655

FIGURE 6

pTG5913

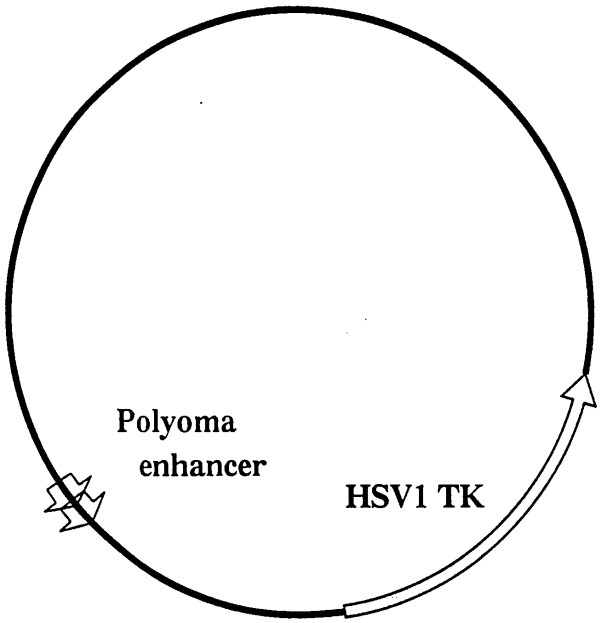

FIGURE 7

pTG8512

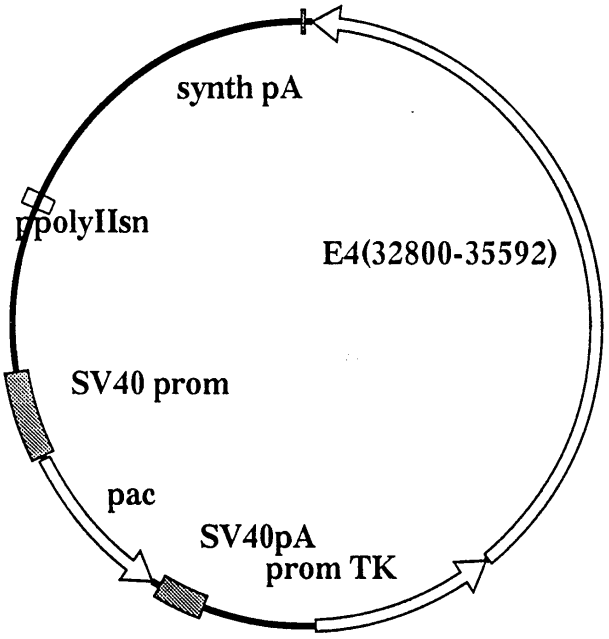

FIGURE 8

pTG8513

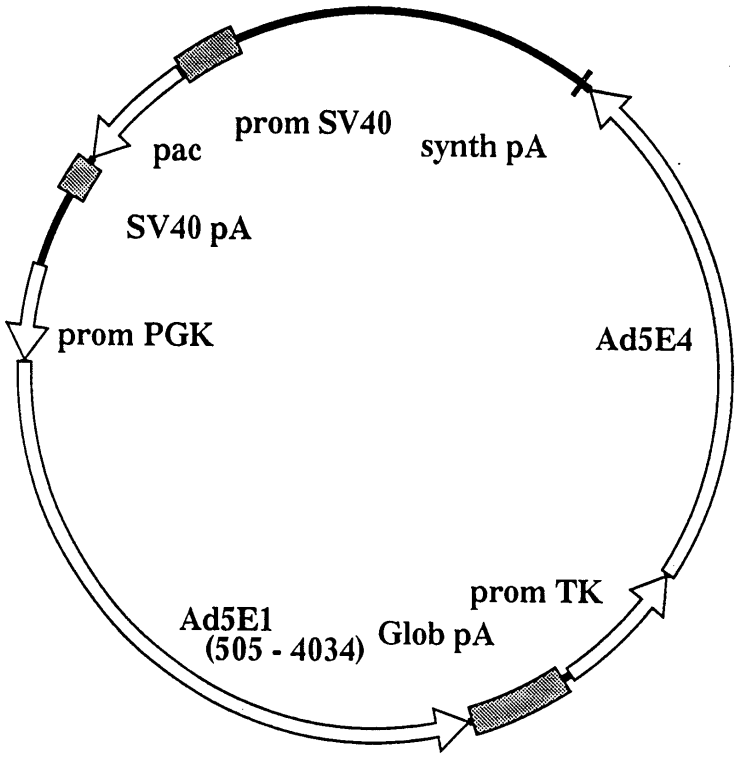

FIGURE 9

pTG8514

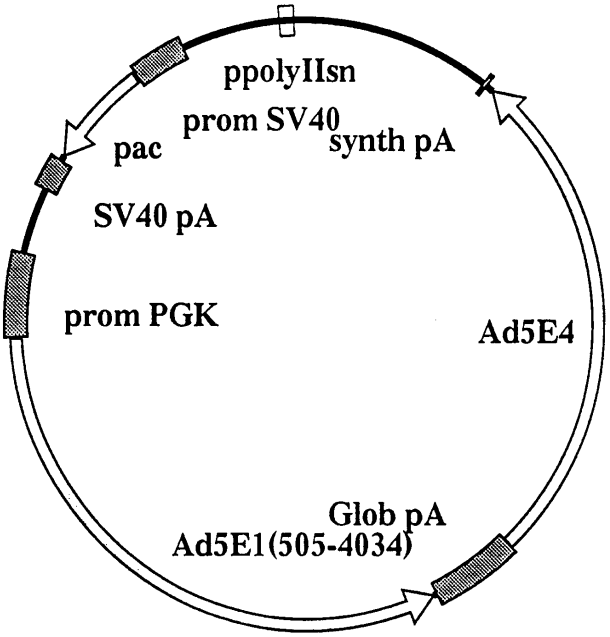

FIGURE 10

pTG8515

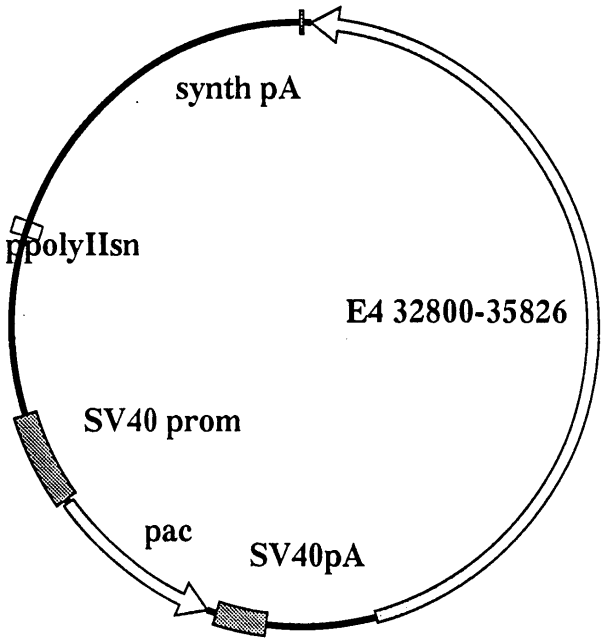

FIGURE 11

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/FR 94/00624

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/86 C12N15/34 C12N5/10 A61K48/00 C12N15/12
C12N7/04 C12N15/23 A61K39/235 C12N15/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY vol. 63, no. 6 , June 1989 pages 2709 - 2717 BABISS, L.E. 'The cellular transcription factor E2F requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression' see page 2715, column 2, line 53 - line 54 see page 2716, column 1, line 6 - line 9 ---	1,2
A	HUMAN GENE TRANSFER vol. 219 , 1991 pages 51 - 61 STATFORD-PERRICAUDET, L. & PERRICAUDET, M. 'Gene transfer into animals: the promise of adenovirus' see page 58, paragraph 6 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 1994	05. 09. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/FR 94/00624

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL. vol. 68, no. 1 , 10 January 1992 , CAMBRIDGE, NA US pages 143 - 155 ROSENFELD, M.A. ET AL. 'In vivo transfer of the human cystic fibrosis transmembrane conductance gene to the airway epithelium' see the whole document ---	9-11
A	WO,A,93 06223 (CNRS) 1 April 1993 see claim 3 ---	1
E	WO,A,94 12649 (GENZYME CORPORATION) 9 June 1994 see the whole document -----	1-10,15, 24,26, 27, 30-32, 42,43, 46,49, 50,52-55

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR 94/00624

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 54 is directed to a method for treatment of the human or animal body, the research has been carried out and based on the the alleged effects of the product (composition)

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte. ɔnal Application No
PCT/FR 94/00624

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9306223	01-04-93	FR-A-	2681786	02-04-93
		AU-A-	2790292	27-04-93
		EP-A-	0559884	15-09-93
		JP-T-	6502771	31-03-94
WO-A-9412649	09-06-94	NONE		

RAPPORT DE RECHERCHE INTERNATIONALE

Den Internationale No
PCT/FR 94/00624

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 5 C12N15/86 C12N15/34 C12N5/10 A61K48/00 C12N15/12
C12N7/04 C12N15/23 A61K39/235 C12N15/31

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 5 C12N C07K A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	JOURNAL OF VIROLOGY vol. 63, no. 6 , Juin 1989 pages 2709 - 2717 BABISS, L.E. 'The cellular transcription factor E2F requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression' voir page 2715, colonne 2, ligne 53 - ligne 54 voir page 2716, colonne 1, ligne 6 - ligne 9 --- -/--	1,2

[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

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Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
24 Août 1994	05. 09. 94

Nom et adresse postale de l'administration chargée de la recherche internationale	Fonctionnaire autorisé
Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

Formulaire PCT/ISA/210 (deuxième feuille) (juillet 1992)

RAPPORT DE RECHERCHE INTERNATIONALE

Den . Internationale No
PCT/FR 94/00624

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	HUMAN GENE TRANSFER vol. 219 , 1991 pages 51 - 61 STATFORD-PERRICAUDET, L. & PERRICAUDET, M. 'Gene transfer into animals: the promise of adenovirus' voir page 58, alinéa 6 ---	1
A	CELL. vol. 68, no. 1 , 10 Janvier 1992 , CAMBRIDGE, NA US pages 143 - 155 ROSENFELD, M.A. ET AL. 'In vivo transfer of the human cystic fibrosis transmembrane conductance gene to the airway epithelium' voir le document en entier ---	9-11
A	WO,A,93 06223 (CNRS) 1 Avril 1993 voir revendication 3 ---	1
E	WO,A,94 12649 (GENZYME CORPORATION) 9 Juin 1994 voir le document en entier -----	1-10,15, 24,26, 27, 30-32, 42,43, 46,49, 50,52-55

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RAPPORT DE RECHERCHE INTERNATIONALE

.emande internationale n°
PCT/FR94/00624

Cadre I Observations - lorsqu'il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (suite du point 1 de la première feuille)

Conformément à l'article 17.2)a), certaines revendications n'ont pas fait l'objet d'une recherche pour les motifs suivants:

1. [X] Les revendications n°s
se rapportent à un objet à l'égard duquel l'administration n'est pas tenue de procéder à la recherche, à savoir:

Remarque: Pour autant que la revendication 54 concerne une methode de traitement du corps humain/animal, la recherche a ete effectuee et basee sur les effets imputes au produit (a la composition)

2. [ ] Les revendications n°s
se rapportent à des parties de la demande internationale qui ne remplissent pas suffisamment les conditions prescrites pour qu'une recherche significative puisse être effectuée, en particulier:

3. [ ] Les revendications n°s
sont des revendications dépendantes et ne sont pas rédigées conformément aux dispositions de la deuxième et de la troisième phrases de la règle 6.4.a).

Cadre II Observations - lorsqu'il y a absence d'unité de l'invention (suite du point 2 de la première feuille)

L'administration chargée de la recherche internationale a trouvé plusieurs inventions dans la demande internationale, à savoir:

1. [ ] Comme toutes les taxes additionnelles ont été payées dans les délais par le déposant, le présent rapport de recherche internationale porte sur toutes les revendications pouvant faire l'objet d'une recherche.

2. [ ] Comme toutes les recherches portant sur les revendications qui s'y prêtaient ont pu être effectuées sans effort particulier justifiant une taxe additionnelle, l'administration n'a sollicité le paiement d'aucune taxe de cette nature.

3. [ ] Comme une partie seulement des taxes additionnelles demandées a été payée dans les délais par le déposant, le présent rapport de recherche internationale ne porte que sur les revendications pour lesquelles les taxes ont été payées, à savoir les revendications n°s:

4. [ ] Aucune taxe additionnelle demandée n'a été payée dans les délais par le déposant. En conséquence, le présent rapport de recherche internationale ne porte que sur l'invention mentionnée en premier lieu dans les revendications; elle est couvertes par les revendications n°s:

Remarque quant à la réserve

[ ] Les taxes additionnelles étaient accompagnées d'une réserve de la part du déposant.

[ ] Le paiement des taxes additionnelles n'était assorti d'aucune réserve.

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Den : Internationale No
PCT/FR 94/00624

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)		Date de publication
WO-A-9306223	01-04-93	FR-A-	2681786	02-04-93
		AU-A-	2790292	27-04-93
		EP-A-	0559884	15-09-93
		JP-T-	6502771	31-03-94
WO-A-9412649	09-06-94	AUCUN		