# VECTORS EXPRESSING A HUMAN INTERFERON FOR THE TREATMENT OF AIDS

The subject of the present invention is a cassette containing a DNA sequence encoding a human IFN whose expression is placed under the control of elements which can be regulated by the human immunodeficiency virus (HIV). The present invention finds a useful application in the treatment or the prevention of infections caused by HIV, especially by gene therapy.

HIV, the etiological agent for AIDS (acquired immunodeficiency syndrome), infects preferably a subpopulation of T lymphocytes (helper T) which are responsible for amplifying the immune response. Consequently, the disease develops into a cell immunity deficiency, thereby making the sick individuals particularly sensitive to opportunistic infections. Up until now, no treatment has proved completely satisfactory in spite of the many efforts which have been invested in the development of new antiviral molecules. These difficulties are without doubt due to the particular complexity of the HIV virus and its capacity to repress the immune defence systems in the infected cells.

HIV is a retrovirus belonging to the lentivirus family. Its genetic material, consisting of an RNA molecule, is encapsidated in a capside of protein nature, which is itself surrounded by a viral envelope. In addition to the structural genes (*gag*, *pol* and *env*) which encode the capside proteins, reverse transcriptase and the envelope protein respectively, the HIV genome comprises regulatory genes which control viral replication. Among the latter, there may be mentioned the *tat* and *rev* genes which encode the TAT and REV proteins respectively.

The TAT protein has a transactivating role in the expression of the HIV genes as a whole (Arya et al., 1985, Science, *229*, 69-73). It seems that TAT is involved both at a transcriptional and posttranscriptional level. It interacts specifically with a short nucleotide sequence called TAR (for Transactivation Responsive

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Region) sequence, and localized at the 5′ end of the genome and the viral transcripts (position +1 to +80; +1 representing the site of initiation of transcription) (Rosen et al., 1985, Cell, *41*, 813-823).

The REV protein enhances transport towards the cytoplasm of the messenger RNAs (mRNAs) encoding the structural proteins, so as to be translated therein. For its part, REV specifically recognizes an RRE sequence (for REV Responsive Element) on the mRNAs. The latter is localized in the *env* gene near another so-called CRS sequence (*Cis*-acting Repression Sequence), which is involved in the nuclear retention of the mRNAs carrying it. It is assumed that the binding of the REV protein at the level of its RRE target sequence has the effect of counterbalancing the inhibitory effect of CRS on the passage of the larger viral mRNAs into the cytoplasm.

The sequences essential for the transcription of the viral genes are localized at the two ends of the genome at the level of the LTRs (Long Terminal Repeat). Whereas the 5′ LTR contains the promoter sequences as well as the TAR sequences, the 3′ LTR comprises the sequences involved in the termination of transcription.

Generally, when a cell is infected by a virus, it secretes interferons (IFNs) in response to the viral infection. This is a group of proteins exerting antiviral effects and which is classified into three types $\alpha$, $\beta$ and $\gamma$. There are more than twenty subtypes of $\alpha$ IFN; the $\beta$ and $\gamma$ IFNs seem to be unique.

Paradoxically, the synthesis of cellular (endogenous) IFNs is repressed in HIV-infected cells. This repression prevents the HIV infected cells and neighboring cells from effectively defending themselves against viral infection (Mark, 1992, AIDS Res. Hum. Retrovirus, *8*, 199-207).

It has now been found that the introduction, into a cell, of a gene encoding a human IFN (exogenous IFN) whose expression can be induced by the TAT and/or REV viral proteins allows the genetically modified cell to defend itself in an infectious context. The aim of the

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present invention is to propose different human IFN expressing vectors which can be regulated by the HIV TAT and/or REV proteins. This approach takes advantage of the replication characteristics of the HIV virus. Thus, in the event of an infection, the synthesis of the cellular IFNs is inhibited but the synthesis of the exogenous IFNs is induced by the HIV virus itself, thus reconstituting an effective defence system against it. The use of an inducible expression system avoids the problems of cell toxicity in the noninfected cells.

Accordingly, the subject of the present invention is an expression cassette comprising at least one DNA sequence encoding a human IFN, placed under the control of elements capable of ensuring its expression in a mammalian cell, characterized in that said elements can be regulated by the human immunodeficiency virus (HIV).

A DNA sequence in use in the present invention can encode an $\alpha$, $\beta$ or $\gamma$ type human IFN. This may be the sequences disclosed in the prior art and especially in Shaw et al. (1983, Nucleic Acid Res., *11*, 555-573) for IFN$\alpha$, Higashi et al. (1983, J. Biol. Chem., *258*, 9522-9529) for IFN$\beta$ and Gray et al. (1982, Nature, *295*, 503-508) for IFN$\gamma$. These sequences can be isolated from the gene or the cDNA. Sequence encoding a human IFN is also understood to mean a sequence encoding an IFN analog. "Analog" is understood to mean a molecule which would have an amino acid sequence which is slightly different from a native IFN but which would preserve most of its antiviral function. In practice, such an analog can be obtained by deletion, substitution and/or addition of one or more amino acids of the native protein or of a fragment thereof. A person skilled in the art knows the techniques which make it possible to carry out these modifications without abolishing the biological function of the protein.

The elements ensuring the expression of a DNA sequence in use within the framework of the present invention are conventional elements allowing the transcription of the DNA sequence into mRNA and the transla-

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tion of the latter into protein, such as for example a promoter and the codons for initiation and termination of translation. In addition, a cassette according to the invention comprises sequences which can be regulated by the HIV virus.

Generally, a promoter which is functional in a mammalian cell, preferably in a human cell, is chosen. It may be derived from any eukaryotic gene or from a viral genome and be of ubiquitous or regulatable nature, especially in response to certain tissue-specific or event-specific cell signals. The choice of the promoter is very wide and within the capability of persons skilled in the art. However, the promoter of the murine PGK (PhosphoGlycerate kinase) and $\beta$-actin genes or the SV40 (Simian Virus 40) promoter is advantageously used.

According to a preferred embodiment, the sequences which can be regulated by HIV consist of a TAR and/or RRE/CRS type sequence. A TAR type sequence interacts with the TAT protein to activate the expression of the genes under its control. For its part, an RRE/CRS type sequence interacts with the REV protein so as to enhance the transport of mRNAs into the cytoplasm. It is indicated that it naturally contains an acceptor splicing site. In accordance with the aims pursued by the present invention, these sequences may be slightly different from the native TAR and RRE/CRS sequences (as published by Wain-Hobson et al., 1985, Cell, *40*, 9-17), provided however that they are typical of interacting with the viral TAT and REV proteins.

As regards a TAR type sequence, the latter is inserted in 3′ of the promoter and, most preferably, in the +1 position (+1 being the site of initiation of transcription). In this context, it may be advantageous to use the HIV 5′ LTR which naturally comprises a promoter which is functional in mammalian cells, followed by the TAR sequence (in the +1 to +80 position). It is also possible to use a portion of it, which comprises the minimum promoter sequences (Sp1, CAAT box and TATA box sequences) and the TAR sequence. The name miniLTR denotes

a 200 bp fragment corresponding to the 3′ end of the LTR and extending from the -120 to +80 positions of the viral genome.

According to another approach, a cassette according to the invention comprises a conventional promoter and an RRE/CRS type sequence capable of interacting with the HIV REV viral protein. Thus, in a noninfected cell, the mRNAs will be blocked in the nucleus under the action of CRS. On the other hand, as soon as the viral infection and the synthesis of REV have taken place, they will be transported into the cytoplasm where they will be translated into INF. Preferably, the RRE/CRS sequence is introduced in 3′ of the DNA sequence encoding an IFN.

According to another variant, the expression of a DNA sequence in use in the present invention may be subjected to a double regulation by TAT and REV, thereby making it possible to avoid the problems of escaping expression which are commonly observed with certain promoters. In this case, an expression cassette according to the invention may comprise a TAR type sequence and an RRE/CRS type sequence such that in the event of escaping expression, the few mRNAs produced will remain blocked in the nucleus. Only the penetration of HIV and the synthesis of the TAT and REV viral proteins will be able to trigger the synthesis of IFN.

Moreover, an expression cassette according to the invention may, in addition, contain other elements which contribute to the expression of the DNA sequence encoding IFN, especially an intron, suitable splicing signals and elements for termination of transcription (polyadenylation signal). The choice and the localization of these elements is within the capability of persons skilled in the art.

By way of preferred examples, there may be mentioned a cassette comprising from 5′ to 3′:

(1) The HIV 5′ LTR, a DNA sequence encoding an IFN and the SV40 polyadenylation signal;

(2) The HIV miniLTR, a DNA sequence encoding an IFN and the SV40 polyadenylation signal;

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(3) The PGK promoter, the TAR sequence, a DNA sequence encoding an IFN and the SV40 polyadenylation signal;

(4) The β-actin promoter, the TAR sequence, a DNA sequence encoding an IFN and the SV40 polyadenylation signal;

(5) The SV40 promoter, a DNA sequence encoding an IFN, an RRE/CRS sequence and the SV40 polyadenylation signal;

(6) The HIV 5' LTR, a DNA sequence encoding an IFN, the RRE/CRS sequence and the SV40 polyadenylation signal;

(7) The HIV miniLTR, a DNA sequence encoding an IFN, the RRE/CRS sequence and the SV40 polyadenylation signal; and

(8) the HIV miniLTR, a donor splicing site (of the HIV *gag* gene), a DNA sequence encoding an IFN, the RRE/CRS sequence and the SV40 polyadenylation signal.

The invention also extends to the vectors comprising an expression cassette according to the invention. Such vectors are well known to the person skilled in the art as well as the methods for constructing them and propagating them. Viral vectors derived from retroviruses, herpes viruses, adenoviruses or adenovirus-associated viruses will be advantageously used. Of course, it is also possible to use synthetic vectors. Within the framework of the invention, replication-defective vectors, and especially retroviral vectors are particularly preferred. According to an advantageous embodiment, a cassette according to the invention is positioned in the reverse orientation relative to the LTRs of the retroviral vector, so as to position the promoters in the opposite direction in order to avoid interference. Of course, a vector according to the invention may, in addition, comprise a gene encoding a selectable marker, such as for example the neomycin and puromycin acetyl transferase genes which confer resistance to the antibiotics G418 and puromycin. The invention also covers the viruses and viral vector particles

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obtained by transfecting the vector according to the invention into an encapsidation, especially amphotropic, cell line producing the structural proteins of the virus in question.

The invention also relates to a eukaryotic cell comprising an expression cassette or a vector according to the invention. The latter may be introduced either *in vitro* into a cell collected from the patient or directly *in vivo*. The cell is advantageously a human cell and, preferably, a hematopoietic cell line (stem cells or primary lymphocytes).

The invention also relates to the therapeutic use of an expression cassette, of a vector or of a cell according to the invention, for the manufacture of a medicinal product intended for the prevention or treatment of viral infections and, in particular, of infections caused by HIV.

The invention also relates to a pharmaceutical composition comprising, as therapeutic or prophylactic agent, an expression cassette, a vector or a cell according to the invention, in combination with a pharmaceutically acceptable vehicle.

A pharmaceutical composition according to the invention may be manufactured in a conventional manner. In particular, a therapeutically effective quantity of such a therapeutic or prophylactic agent is combined with a carrier, a diluent or an acceptable adjuvant. It may be administered by any conventional route in use in the art. The administration may take place in a single dose or in a dose which is repeated after a certain time interval. The quantity to be administered will be chosen as a function of different criteria, in particular the route of administration, the patient, the progression of the disease and the duration of the treatment. As a guide, a pharmaceutical composition according to the invention contains $10^3$ to $10^{14}$ pfu (plaque forming unit), advantageously $10^5$ to $10^{13}$ pfu, preferably $10^6$ to $10^{12}$ pfu and, most preferably, $10^7$ to $10^{10}$ pfu of particles of viral vector.

Moreover, the invention relates to a method of

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treating viral, and especially HIV, infections, according to which a therapeutically effective quantity of an expression cassette, a vector or a cell according to the invention is administered to a patient requiring such a treatment. According to a first therapeutic procedure, they can be administered directly *in vivo*, for example intravenously. A somatic gene therapy procedure will be preferably adopted which consists in collecting bone marrow stem cells or peripheral blood lymphocytes from a patient, transfecting them with a vector according to the invention, culturing them *in vitro* according to prior art techniques and administering them to the patient. As a guide, it may be advantageous to use $10^6$ to $10^{10}$ cells per kg, preferably $10^7$ to $10^9$ and preferably $5 \times 10^7$ to $5 \times 10^8$.

Naturally, this procedure may be subject to numerous variants. In particular, it may be advantageous to combine at least two cassettes, vectors or cells according to the invention allowing the expression of an IFN of different types, for example an IFN$\alpha$ and an IFN$\beta$.

The invention is illustrated below with reference to the following figures.

Figure 1 schematically represents the vector pTG4313 which allows the expression of a DNA sequence encoding IFN$\alpha$ under the control of the HIV LTR.

Figure 2 represents the vectors pTG4344 which coexpresses the IFN$\alpha$ gene and the puromycin selectable gene.

Figure 3 schematically represents the retroviral vector pTG4379 for transferring an IFN gene and its TAT-inducible expression.

Figure 4 shows the progression of the infection in U937 cells infected with HIV III B and transfected with the vectors for expressing IFN$\alpha$ (□), $\beta$ (♦) and $\gamma$ (◇), compared with the control consisting of nontransfected cells (■).

Figure 5 illustrates the retroviral vector pTG6324 comprising the cassettes for expressing IFN$\alpha$ (under the control of the HIV miniLTR) and the *neo*

selectable gene (under the control of the retroviral 5' LTR).

Figure 6 illustrates the progression of the infection in $CD8^-$ human PBLs transduced by vector pTG6327 (Δ) then infected by the HIV III B virus, compared with the nontransduced cells (♦).

EXAMPLES

All the cloning operations detailed below are carried out according to conventional molecular biology techniques (Maniatis et al., 1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY). These steps are performed in the *Escherichia coli (E. coli)* 5K, XL1-Blue or 1106 strain.

The human genes encoding the α and β IFNs were cloned by PCR (Polymerase Chain Reaction) from the genomic DNA of lymphoid cells, for example CEM-A3 cells (derived from a CEM line available at the ATCC under number CCL119). Such libraries are commercially available. The amplification of the DNA fragments by PCR may be carried out with the aid of commercial kits according to the manufacturer's specifications or according to the technology described in PCR Protocols-A (published by Innis, Gelfand, Sninsky and White, Academic Press Inc) with the aid of primers complementary to the published sequences. The human gene encoding IFNγ is obtained from the pTG11 described in the French patent application published under number 2,583,429. Suitable restriction sites were introduced upstream and downstream of the initiator ATG and of the stop codon of the different IFN genes so as to be able to facilitate the subsequent cloning steps. Moreover, the 5' protruding restriction sites may be converted into blunt sites by treating with the large Klenow fragment of *E. coli* DNA polymerase whereas the 3' protruding sites are treated with T4 polymerase.

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EXAMPLE 1: Construction of interferon expression vectors inducible by the HIV TAT protein and in which the selectable gene consists of the puromycin gene.

A. *Nonviral vectors for the expression of IFN under the control of the HIV promoter.*

The construction of vectors in which the expression of the IFN$\alpha$, $\beta$ and $\gamma$ genes is placed under the control of the HIV 5' LTR is described. These vectors were constructed from the vector pTG1322 described in Mehtali (1992, AIDS and Human Retroviruses, *8*, 1959-1965) comprising a heterologous gene placed under the control of the HIV 5' LTR (725 bp *Xho*I-*Hind*III fragment corresponding to the -645 to +80 positions of the HIV genome). This vector also carries the intron and the SV40 virus polyadenylation signal. It is digested with *Hind*III treated with Klenow and then digested with *Sma*I.

Into the pTG1322 thus treated, there is introduced the IFN$\alpha$ gene in the form of a fragment delimited by the *Sma*I and *Eco*RI sites, treated with Klenow and pTG4313 is generated (Figure 1). pTG4314 is obtained by inserting the *Sma*I-*Eco*RV fragment carrying the IFN$\beta$ gene into pTG1322 obtained as above. pTG4315 is generated by inserting the IFN$\gamma$ gene in the form of a *Hpa*I-*Pvu*II fragment between the same PTG1322 sites.

A selectable gene conferring the resistance to puromycin (puromycin R), in the form of a Klenow-treated 1.2 kb *Bam*HI-*Bgl*II fragment, is introduced into the *Eco*RI site of these different vectors for the expression of an IFN. This fragment contains the SV40 virus early promoter, the puromycin R gene and the SV40 polyadenylation signal. The vectors pTG4344 (represented in Figure 2), pTG4345 and pTG4346 are obtained which allow coexpression of the puromycin R selectable gene and the IFN$\alpha$, $\beta$ or $\gamma$ gene respectively.

B. *Retroviral vector for the transfer and expression of the IFN genes.*

The vector forming the basis for the constructs is pLXSP which is derived from pLXSN (Miller and Rossman, 1989, Biotechniques, 7, 980-988) after replacement of the

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neomycin selectable gene by the puromycin R gene and of the MSV (Myelo Sarcoma Virus) 3′ LTR by MPSV (Myelo Proliferative Sarcoma Virus) 3′ LTR.

A 200 bp *Sca*I-*Hind*III fragment corresponding to the miniLTR (-120 to +80 position) is isolated from the HIV genome and then introduced upstream of each of the IFN genes. The SV40 polyadenylation signal is then introduced in 3′ of the genes. The vector thus obtained is digested with *Bgl*II, treated with Klenow. The fragment containing the expression cassette "miniLTR-IFN-polyadenylation signal" is introduced between the *Hpa*I-*Bgl*II sites of the retroviral vector pTG4056. The clones in which this fragment is positioned in the reverse orientation relative to the 5′ and 3′ LTRs are selected. pTG4056 is obtained from pLXSP following the deletion of the expression block SV40 promoter-puromycin R and the reinsertion of the puromycin R gene in the correct orientation so as to be placed under the control of the 5′ LTR.

The retroviral vectors pTG4392, pTG4391 and pTG4379 (Figure 3) are obtained which allow the transfer and the expression of the sequences encoding IFNs $\alpha$, $\beta$ and $\gamma$ respectively. They may be transfected into an amphotropic encapsidation cell line, for example the PA317 (Miller et al., 1986, Mol. Cell., Biol. *6*, 2895-2902), Gp Env. Am (Markowitz et al., 1988, Virology, *167*, 400-406) or PG13 (Miller et al., 1991, J. Virol, *65*, 2220-2224) line. The next day, the transfected cells are placed in a selective medium (puromycin 6 $\mu$g/ml). The resistant cells are isolated which will constitute lines producing amphotropic particles capable of infecting human cells, from which a viral stock can be constituted which is capable of being used in an anti-AIDS gene therapy.

*C. TAT-regulatable vectors in which the expression of IFN is directed by a eukaryotic promoter.*

1. Murine PGK promoter

The TAR sequence (corresponding to nucleotides +1 to +80 of the HIV genome) is generated by chemical

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synthesis. Then, the double-stranded oligonucleotide thus obtained is introduced by insertional mutagenesis into the +1 position of the murine PGK promoter whose sequence is published in Adra et al. (1987, Gene, *60*, 65-74). The fragment containing the sequence encoding human IFN $\alpha$, $\beta$ or $\gamma$ followed by the SV40 polyadenylation signal is inserted downstream of the TAR sequence.

Finally, the expression cassette "PGK promoter-TAR-IFN-polyA" is introduced into the retroviral vector pLXSP. Of course, to each IFN$\alpha$, $\beta$ or $\gamma$ sequence corresponds a retroviral vector. As above, an amphotropic encapsidation line producing each type of viral particles is established.

2. Murine $\beta$-actin promoter

The preceding example is reproduced with the following variant:

The synthetic TAR sequence is introduced into the +1 position of the rat $\beta$-actin promoter whose sequence is described in Nudel et al., (1983, Nucleic Acids Res., *11*, 1759-1771).

<u>EXAMPLE 2: Construction of vectors for the expression of a human IFN which are inducible by the HIV TAT protein and in which the selectable gene consists of the neomycin gene and establishment of corresponding amphotropic lines.</u>

*A. Construction of pTG6324 for the expression of IFN$\alpha$.*

In the first place, the HIV miniLTR is isolated from the viral genome after *Sca*I-*Hind*III digestion, inserted into any plasmid and removed in the form of a *Sma*I-*Hind*III fragment and then cloned between the same sites of the vector pTG2359. The latter corresponds to the cloning of the SV40 virus polyadenylation site into the plasmid p Bluescript KS (Stratagene). pTG4347 is obtained into which a *Sma*I-*Hpa*I fragment carrying the sequences encoding IFN$\alpha$ is introduced after digestion with *Bal*I.

Finally, the expression cassette "miniLTR-IFN$\alpha$-SV40 polyA" is purified from the vector pTG4355 obtained in the preceding stage in the form of a *Bam*HI-*Bgl*II

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fragment, which is cloned into the *Bam*HI site of pTG6320 to give pTG6324 (Figure 5). The latter therefore comprises the MSV LTR which directs the expression of the *neo* selectable gene, followed by the cassette "miniLTR-IFN$\alpha$-SV40 polyA" positioned in the reverse orientation so as to avoid interference phenomena between the promoters and finally the MPSV retroviral 3' LTR. As a guide, pTG6320 is obtained from pLXSP following the deletion of the cassette for the expression of the puromycin gene and replacement with the neomycin gene (Colbère - Garapin et al., 1981, J. Mol. Biol., *150*, 1-14).

*B. Construction of pTG6328 for the expression of IFN$\gamma$.*

The cloning strategy is completely similar to that which is described above. The vector pTG6311 is obtained from the insertion of a *Sma*I-*Hpa*I fragment carrying the sequence encoding IFN$\gamma$ into the *Bal*I site of pTG4347. Then the retroviral vector pTG6328 is obtained by cloning the *Bam*HI-*Bgl*II fragment purified from pTG6311 into the vector pTG6320 previously digested with *Bam*HI. The latter is the equivalent of pTG6324 with the difference that it carries the IFN$\gamma$ gene in place of the IFN$\alpha$ gene.

*C. Construction of the retroviral vector pTG6327 for the expression of IFN$\beta$.*

The *Sma*I-*Hind*III fragment carrying the HIV miniLTR is inserted between the same pTG4356 sites, to generate pTG6312. The vector pTG4356 corresponds to the vector pTG2359 into which the IFN$\beta$ gene (*Sma*I-*Eco*RV fragment) has been cloned (*Bal*I site). As above, the cassette for the expression of IFN$\beta$ is purified from pTG6312 after digestion with *Bam*HI-*Bgl*II and then sub-cloned into the *Bam*HI site of pTG6320 to give pTG6327.

*D. Establishment of amphotropic lines for the production of infectious viral particles*

Stocks of infectious viral particles can be prepared from each of the preceding vectors pTG6324, pTG6327 and pTG6328. For that, the latter are conventionally transfected into an ecotropic encapsidation line, for example the GP + E 86 line (Markowitz et al., 1988, J. Virol., *62*, 1120-1124). 48 h after the

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transfection, the cells are cultured in selective medium (G418 1 mg/ml).

The mixture of G418-resistant clones is maintained for 3 additional days in a selective medium. The culture supernatant is harvested so as to conventionally infect an amphotropic encapsidation line such as the PA317 line. After selection, the lines thus obtained will constitute the cells producing infectious viral particles which allow the transfer and expression of different IFNs within the context of a human gene therapy.

Their capacity to produce an active IFN can be evaluated in the culture supernatants by the method described in The Interferon System (W.E. Stewart, 1979, Ed: Springer-Verlag Wien, New York). This functionality test, when carried out on the cells generated in the preceding step, gives a low IFN level in the three cases. On the other hand, when the cells are transfected transiently by the plasmid pHMG-TAT, the level measured is high and reaches a few hundreds of units per ml. pHMG-TAT is a vector for the expression of the native TAT protein and results from the cloning of the sequence encoding the latter in the vector pHMG (Mehtali et al., 1990, Gene, *91*, 179-184).

Moreover, it may be advantageous to isolate producing clones. This is conventionally performed by the clonal dilution technique (0.3 cell per well). A clone which is a good producer of virions with a titre of 5 x $10^6$ to $10^7$ pfu/ml is selected after infecting the murine cells NIH-3T3 (ATCC CRL 1658) with an aliquot of clone culture supernatant. Its capacity to produce IFN is checked as indicated above. A clonal dilution is again carried out starting with the preceding clones so as to generate subclones. A subclone which is a good producer of virions and of IFN is selected for each of them after titration and assay of IFN.

The subclones are frozen before use for the purpose of gene therapy.

<u>EXAMPLE 3: Construction of vectors for the expression of a human IFN which are inducible by the HIV REV protein.</u>

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A retroviral vector is described by inserting into the vector pLXSP and in the reverse orientation relative to the LTRs an expression cassette comprising from 5′ to 3′:

- the SV40 virus promoter;
- the DNA sequence encoding human IFN $\alpha$, $\beta$ or $\gamma$;
- the RRE/CRS sequence. The latter is isolated from the genome of HIV-1 Lai isolate in the form of a *Bgl*II-*Bam*HI fragment (extending from nucleotides 7178 to 8032 of the sequence as published by Wain-Hobson et al., *supra*). This fragment may be treated with Klenow before being introduced into a suitable restriction site; and
- the SV40 polyadenylation signal.

As above, viral stocks can be constituted after transfection into an amphotropic encapsidation line.

EXAMPLE 4: Construction of vectors for the expression of a human IFN which are inducible by the HIV TAT and REV proteins.

There is inserted into the vectors pTG4392, pTG4391 and pTG4379 the *Bam*HI-*Bgl*II fragment treated with Klenow and carrying the HIV RRE/CRS sequence between the sequence encoding one of the IFNs and the SV40 polyadenylation signal.

Furthermore, the vectors thus obtained may be modified by inserting a donor splicing site between the HIV LTR and the IFN gene.

As above, it is possible to generate an amphotropic line producing infectious particles corresponding to each of the vectors thus obtained.

EXAMPLE 5: Resistance to viral infection of the cell lines transfected with the vectors for the expression of IFN which are inducible by TAT.

The experiments described below are carried out in cell lines which can be naturally infected with HIV, such as:

- the U937 line (ATCC CRL1593) derived from human monocytes; and
- the CEM-A3 line derived from CD4+ human lympho-

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cytic cells (ATCC CCL119).

In practice, the cells are cultured according to the supplier's recommendations and transfected by electroporation of at least 20 $\mu$g of DNA with the aid of an appropriate apparatus (Gene Pulser Biorad, voltage 210 V, capacitance 960 $\mu$F).

In a first experiment, pTG4314 is co-electroporated with the vector pLXSP into the U937 cells ($2\times10^7$ cells) in a 20:1 DNA concentration ratio. The cells are reinoculated at 37°C in the presence of 5 % $CO_2$ and the next day, they are placed in selective medium (puromycin 0.5 $\mu$g/ml). The cells resistant to the antibiotic are selected.

Their resistance to infection is tested by infecting them with a HIV preparation at a high $TCID_{50}$ (at least 1000). The $TCID_{50}$ is determined according to the method described in Reed et al., (1938, Am. J. Hyg., *27*, 493-497), on cell lines which can be infected with HIV, such as the CEM cells. It corresponds to the dose at which 50 % of the cells are infected and 50 % are not. It is checked for each batch of HIV used.

For this, a fraction of the electroporated U937 cell culture, corresponding to about $10^6$ cells, is collected and centrifuged at low speed. After washing, the cellular pellet is taken up in 200 $\mu$l of culture medium in the absence of serum. Then the cells are treated with different dilutions of the viral preparation. The infection is allowed to continue for 1 hour at 37°C.

The cells are then washed twice in culture medium and inoculated in 5 ml of the same medium, supplemented with 10 % FCS (fetal calf serum). The medium is renewed 2 or 3 times per week. The propagation of the virus is evaluated by measuring the reverse transcriptase activity at regular intervals in the culture supernatant according to the method described in Spire et al. (1985, Lancet, i, 188-189).

A significant decrease (up to 85 %) is observed in the reverse transcriptase activity over 14 days post-

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infection compared with the control U937 cells not electroporated with an interferon expression vector.

The experiment is repeated this time with U937 cells initially eletroporated with pTG4344, pTG4345 or pTG4346. In the three cases, an even greater decrease is observed in the reverse transcriptase activity over 21 days post-infection compared with the nonelectroporated control cells (Figure 4).

EXAMPLE 6: Molecular mechanism of resistance to HIV infection.

The analysis is carried out on U937 cells electroporated and infected *in vitro* with HIV (Figure 5).

The presence of the HIV genome is first determined in a cellular genomic DNA preparation. This is performed by PCR with the aid of suitable primers which allow the amplification of a fragment of the viral *gag* gene. The presence of an amplification band of the expected size indicates that the expression of IFN does not interfere with the integration of the HIV genome in proviral form into the cellular chromosomes.

It was also possible to detect the presence of mRNAs encoding the products of the gag gene by the reverse transcription PCR technique (same primers as above). This suggests that the expression of IFN has no notable effect on the transcription of proviral sequences.

Finally, the presence and the localization of proviral protein gp120 are evaluated by immunofluorescence with the aid of an anti-gp120 antibody (obtained from serum from seropositive patients). It is observed that in cells expressing IFN, the gp120 protein is in intracellular form whereas it should be in submembrane form, in order to allow budding of the viral particles.

As a whole, the results indicate that IFN acts at the level of the late stages of the viral cycle and probably at the level of the virion assembly.

EXAMPLE 7: Method of treatment of AIDS by gene therapy.

Within the framework of a gene therapy applied to man, two types of target cells may be envisaged:

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- the peripheral blood CD4+ lymphocytes which can be collected especially by leukapheresis, and
- the CD34+ hematopoietic stem cells which can be obtained from a bone marrow sample or peripheral blood (mobilization by chemotherapy or by the action of hematopoietic growth factors).

*A. Procedure applicable to the CD4+ cells*

The patient is subjected to blood sample collection or to a leukapheresis. After having removed the red blood cells, the mononuclear cells are isolated by centrifugation on Ficoll and cultured according to prior art techniques. In the first instance, the cells adhering to the plastic of the culture flasks are removed. The cellular suspension is recovered and cultured in flasks coated with anti-CD8 antibody (Immunotech) so as to remove the CD8+ cells. The cellular suspension thus obtained is predominantly composed of CD4+ lymphocytes. Of course, other enriching techniques can also be used.

These are stimulated in order to induce cell division before carrying out the retroviral transduction. The growth factors which can be used for this purpose are varied. As a guide, there may be mentioned a mixture of human anti-CD3 antibodies (Immunotech, about 10 ng/ml) and of IL-2 (Cetus; 100 U/ml) or alternatively PHA (phytohemagglutinin, Sigma, at a concentration of about 5 $\mu$g/ml) and of IL-2.

After 72 hours of culture under these conditions, the cells are transduced with the retroviral vector. Various methodologies are applicable:

- co-culture of the CD4+ cells and of the amphotropic producing cells of Examples 1 to 4.
- co-culture of the two cell types, the CD4+ cells being deposited in the culture wells and the producing cells in a Transwell (Costar)
- infection of the CD4+ cells with an aliquot of culture supernatant of the producing cells based on a m.o.i. (multiplicity of infection) preferably of between 1 and 5.

Generally, the transduction is performed in the

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presence of protamine sulfate (at a concentration of about 5 μg/ml) in order to enhance the attachment of the virus to the cell surface.

On the next day, the cell culture is transferred to a selective medium (puromycin in the context of the vectors of Example 1 or G418 in the context of the vectors of Example 2) and continued for 7 to 8 days until the appropriate cell number is obtained. It is indicated that it may be advantageous to use the AIM V medium (Gibco BRL) supplemented with L-glutamine (2 mM) and with IL-2 (100 U/ml) and free of serum.

The capacity of these cells to inhibit the replication of HIV is tested *in vitro*. They are infected with HIV-1 IIIB at a m.o.i. of $10^{-2}$ according to the conventional methodology in the prior art. After washing and reinoculation, the propagation of the virus is evaluated by measuring the reverse transcriptase activity in the culture supernatant (Figure 6). The latter is situated at a very low level during at least 20 days of culture. By way of comparison, in the case of the non-transduced cells, the reverse transcriptase activity increases very strongly up to a maximum from the third day of culture and is rapidly followed by cell death.

*B. Procedure applicable to the CD34+ cells.*

The steps of the purification of the CD34 cells are the following:

- isolation of mononuclear cells from the sample of peripheral blood or of bone marrow by Ficoll centrifugation,
- depletion of the adhering cells, and
- capture of the CD34+ cells by culture in flasks coated with anti-CD34 antibody (Immunotech) or in the presence of beads coated with this antibody. The cells in suspension are removed and the adhering cells are recovered after the action, for example, of papain.

As above, the CD34+ cells thus obtained are stimulated by various growth factors so as to place them in the dividing cycle and thus enhance the retroviral

infection. It is possible to use for this purpose a mixture of IL-3 (about 10 ng/ml), IL-6 (about 50 U/ml) and SCF (Stem cell factor, about 50 U/ml) incorporated into the culture medium for 48 hours before carrying out the transduction according to the procedure indicated above. These factors are commercially available. The cells are then readministered to the patient.

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Claims

1. Cassette comprising at least one DNA sequence encoding a human IFN, placed under the control of elements capable of ensuring its expression in a mammalian cell, characterized in that the said elements can be regulated by the human immunodeficiency virus (HIV).

2. Cassette according to Claim 1, characterized in that IFN is selected from $\alpha$, $\beta$ and $\gamma$ type human IFNs.

3. Cassette according to Claim 1 or 2, characterized in that the elements capable of ensuring expression in a mammalian cell comprise at least one promoter and a TAR and/or RRE/CRS type sequence.

4. Cassette according to Claim 3, characterized in that the TAR type sequence is placed in 3′ of the said promoter.

5. Cassette according to Claim 3 or 4, characterized in that the RRE/CRS type sequence is placed in 3′ of the DNA sequence encoding a human IFN.

6. Cassette according to one of Claims 1 to 5, characterized in that the promoter which is functional in a mammalian cell is selected from the promoters of murine PGK and -actin genes, HIV 5′ LTR and a fragment thereof (miniLTR).

7. Cassette according to one of Claims 1 to 6, characterized in that the the said elements contain, in addition, a donor splicing site.

8. Cassette according to one of Claims 1 to 7, characterized in that the said elements comprise, in addition, a polyadenylation signal.

9. Vector comprising a cassette according to one of Claims 1 to 8.

10. Vector according to Claim 9, characterized in that it is a retroviral vector.

11. Vector according to Claim 10, characterized in that the expression cassette according to one of Claims 1 to 8 is positioned in reverse orientation relative to the LTRs of the said vector.

12. Vector according to one of Claims 9 to 11,

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characterized in that it comprises, in addition, a selectable gene.

13. Vector according to Claim 12, characterized in that the selectable gene confers resistance to puromycin or neomycin.

14. Eukaryotic cell comprising a cassette according to one of Claims 1 to 8 or a vector according to one of Claims 9 to 13.

15. Cell according to Claim 14, characterized in that it is a human cell of the hematopoietic line.

16. Use of a cassette according to one of Claims 1 to 8, of a vector according to one of Claims 9 to 13 or of a cell according to Claim 14 or 15, for the manufacture of a medicinal product intended for the prevention or treatment of viral infections.

17. Use according to Claim 16, intended for the prevention or treatment of infections caused by HIV.

18. Pharmaceutical composition comprising a therapeutically effective quantity of a cassette according to one of Claims 1 to 8, of a vector according to one of Claims 9 to 13 or of a cell according to Claim 14 or 15, in combination with a pharmaceutically acceptable vehicle.

19. Pharmaceutical composition according to Claim 18, characterized in that it comprises $10^3$ to $10^{14}$ pfu of a vector according to one of Claims 9 to 13.

20. Use of at least two cassettes according to one of Claims 1 to 8, vectors according to one of Claims 9 to 13 or cells according to Claim 14 or 15, each expressing a different type of IFN, for simultaneous, separate or staggered use which is intended for the prevention or treatment of viral infections and, in particular, of infections caused by HIV.

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AMENDED CLAIMS

[Received by the International Office on 28 April 1995 (28.04.95); Claims 1-20 replaced by amended Claims 1-19 (2 pages)]

1. Cassette comprising at least one DNA sequence encoding a human IFN, placed under the control of elements capable of ensuring its expression in a mammalian cell, characterized in that the said elements comprise at least one promoter and a TAR and/or RRE/CRS type sequence and can be regulated by the human immunodeficiency virus (HIV).

2. Cassette according to Claim 1, characterized in that IFN is selected from $\alpha$, $\beta$ and $\gamma$ type human IFNs.

3. Cassette according to either of Claims 1 and 2, characterized in that the TAR type sequence is placed in 3′ of the said promoter.

4. Cassette according to one of Claims 1 to 3, characterized in that the RRE/CRS type sequence is placed in 3′ of the DNA sequence encoding a human IFN.

5. Cassette according to one of Claims 1 to 4, characterized in that the promoter which is functional in a mammalian cell is selected from the promoters of murine PGK and $\beta$-actin genes, HIV 5′ LTR and a fragment thereof (miniLTR).

6. Cassette according to one of Claims 1 to 5, characterized in that the said elements contain, in addition, a donor splicing site.

7. Cassette according to one of Claims 1 to 6, characterized in that the said elements comprise, in addition, a polyadenylation signal.

8. Vector comprising a cassette according to one of Claims 1 to 7.

9. Vector according to Claim 8, characterized in that it is a retroviral vector.

10. Vector according to Claim 9, characterized in that the expression cassette according to one of Claims 1 to 7 is positioned in reverse orientation relative to



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the LTRs of the said vector.

11. Vector according to one of Claims 8 to 10, characterized in that it comprises, in addition, a selectable gene.

12. Vector according to Claim 11, characterized in that the selectable gene confers resistance to puromycin.

13. Eukaryotic cell comprising a cassette according to one of Claims 1 to 7 or a vector according to one of Claims 8 to 12.

14. Cell according to Claim 13, characterized in that it is a human cell of the hematopoietic line.

15. Use of a cassette according to one of Claims 1 to 7, of a vector according to one of Claims 8 to 12 or of a cell according to either of Claims 13 and 14, for the manufacture of a medicinal product intended for the prevention or treatment of infections caused by HIV.

16. Pharmaceutical composition comprising a therapeutically effective quantity of a cassette according to one of Claims 1 to 7, of a vector according to one of Claims 8 to 12 or of a cell according to either of Claims 13 and 14, in combination with a pharmaceutically acceptable vehicle.

17. Pharmaceutical composition according to Claim 16, characterized in that it comprises $10^3$ to $10^{13}$ pfu of a vector according to one of Claims 8 to 12.

18. Product containing at least two cassettes according to one of Claims 1 to 7, vectors according to one of Claims 8 to 12 or cells according to either of Claims 13 and 14, each expressing a different type of IFN, as combination product for simultaneous, separate or staggered use in the prevention or treatment of infections caused by HIV.

19. Use of at least two cassettes according to one of Claims 1 to 7, vectors according to one of Claims 8 to 12 or cells according to either of Claims 13 and 14, each expressing a different type of IFN, in simultaneous, separate or staggered use, intended for producing a medicinal product for the prevention or treatment of

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infections caused by HIV.




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