

The invention relates to a recombinant DNA containing a nucleotide sequence coding for a specific polypeptide under the control of an adenovirus promoter, vectors containing this recombinant DNA and eukaryotic cells transformed by this recombinant DNA, the excretion products of these transformed cells and their uses, in particular for the constitution of vaccines.

The human adenoviruses possess a long, linear and double-stranded genome (about 36,000 bp) which codes for at least 30 proteins. The viral cycle occurring during the infection of permissive cells is divided into two phases, early and late. It is known that the four regions of the viral genome expressed in the early phase are designated the E1, E2, E3 and E4 regions, the respective positions of which on the entire viral genome are shown schematically in Fig. 1. The E1 region, situated at the left extremity of the genome is itself divided into two regions E1A and E1B. The passage from the early phase to the late phase, indicated by the replication of the viral DNA, is characterized by a sudden change in the genetic program of the virus. The expression of some early genes is repressed whereas the transcription of the late genes occurs mainly starting from a single promoter, the major late promoter (Fig. 1). Furthermore, a considerable repression of the synthesis of the proteins of the cell host is observed (1).

The genetic organisation of the human adenoviruses of type 2 or 5 (Ad2, Ad5) is sufficiently well known for it to be possible to manipulate their genomes in vitro and its use as expression vector of a foreign gene in an animal cell in culture has already been considered. It is in fact known that the E3 region, which represents 6% of the genome, is not essential in vitro and may thus be replaced in its totality (2). The size of the fragment of foreign DNA which it is possible to insert in the genome of these viruses is large. In fact, the virus may encapsidate a genome, the length of which exceeds 5% of that of the wild-type genome.

The promoter of the early region E1A of an adenovirus, transported into mouse cells in which the replication of the retroviruses is not possible, nonetheless proves to be capable of

exercising its promoter activity in these cells. Thus, it has been possible to express a prokaryotic gene for chloramphenicol-acetyltransferase in such a cell system under the control of the said promoter (Kruczek I. et al (1983), Proc. Natl. Acad. Sci. USA).

5 Various vectors derived from the adenoviruses of type 2 or 5 have thus been constructed. In such recombinants, the foreign gene was expressed under the control of the major late promoter. In some cases this has made it possible to obtain a synthesis of the protein encoded by a foreign gene at a level comparable to that of the late viral proteins (4, 5, 6, 7), with low in vivo translation efficiencies, 10 in particular in view of the efficiencies of transcription of RNAs also observed (6). In addition, the expression of the foreign gene under the control of the late promoter only occurs in the late phase of the viral cycle.

15 The invention results from the observation that the promoter of the early region E1A of the genome of an adenovirus (hereafter designated simply as "E1A promoter") is able to control the expression of a heterologous gene (i.e. foreign with respect to the genes which are normally combined in the adenovirus) or more generally of a 20 heterologous nucleotide sequence coding for a polypeptide sequence whose expression is desired in a viral vector in a particularly efficient manner. Under the conditions defined below, the E1A promoter behaves like a strong promoter, more particularly when the E1A promoter-heterologous coding sequence assembly is inserted into this viral 25 vector.

Hence, the invention relates in a general manner to a recombinant vector for the transformation of eukaryotic cell lines, in particular human or animal, selected from those which can be infected by adenoviruses or the endogenous polymerases of which are capable 30 of recognizing the promoters of the adenoviruses, this vector being, in addition, modified by a nucleic acid insert containing a nucleotide sequence coding for a polypeptide sequence, the expression of which is desired in the said cell lines, characterized in that the said insertion sequence is substituted for the E1A genes normally placed 35 under the direct control of the said early promoter of the E1A region,

this insertion sequence being itself placed under the direct control of this promoter and in that it contains, downstream from the nucleic acid insert, all of the essential sequences of an adenovirus genome which are necessary for the replication of the corresponding adenovirus and which are normally situated downstream from the genes normally under the direct control of the early E1A promoter in the said genome, and in that the above-mentioned nucleotide sequence coding for the polypeptide sequence whose expression is desired is heterologous with respect to the genes of the above-mentioned adenovirus.

Since it is a viral vector, derived from an adenovirus, it is then also possible to profit from the advantage associated with the E1A region of the adenoviruses, namely that its expression is constitutive and continuous throughout the viral cycle (8, 9).

A particularly preferred form of the recombinant DNA according to the invention is characterized by the fact that it contains, downstream from the nucleic acid insert, in the sense of transcription, a defective adenovirus genome comprising nonetheless all of the essential sequences necessary for the replication of the corresponding adenovirus, which are normally situated downstream from genes normally under the direct control of the early promoter E1A in the said genome.

Advantageously, the defective adenovirus genome with which the recombinant DNA conforming to the invention is combined is constituted by a complete adenovirus genome, nonetheless lacking the early part of the E1A region of the viral genome, in particular its 0-2.6% fragment (percentage expressed with respect to the total size of the genome of the adenovirus).

The recombinant DNAs of the invention, combined with vector elements such as those which were mentioned above, constitute in fact vectors containing the said recombinant DNAs. They will also be referred to as "defective recombinant viruses" when the vector elements combined with the recombinant DNA according to the invention are derived from a defective adenovirus genome. These defective recombinant viruses are advantageously used for the transformation of transformable cell lines of higher eukaryotes (in particular of human or animal origin), which themselves bear a distinct sequence of nucleotides capable of

complementing the part of the adenovirus genome which the above-mentioned vector lacks, the said distinct sequence being preferably incorporated in the genome of the cells of the said cell line.

5 As a preferred example of such cell lines, mention may be made of the line 293, the human embryonic kidney line which contains, integrated in its genome, the first eleven percent of the left-hand end of the genome of a Ad5. These latter make it possible to complement defective recombinant viruses which bear deletions in this region (10).

10 The use of such recombinant defective virus vector systems - cells containing a sequence capable of complementing the defective recombinant viruses - is of quite special interest, when the nucleotide sequence contained in the nucleic acid insert of the recombinant DNA codes for a protein which, when it is expressed in a natural cell host under the control of its natural promoter, is excreted into the medium
15 of a culture of this natural cell host.

In this respect, the S gene of the genome of the hepatitis B virus constitutes a nucleotide sequence of special interest, and does so for several reasons. On the one hand, the expression product of the S gene in the cells which express it, the HBsAg (11, 12), is
20 secreted into the cell supernatant in the form of particles which can easily be detected and quantified by radioimmunological assay, which makes possible a precise evaluation of the expression capacity of the viral vector. On the other hand, the invention provides a recombinant viral vector which makes possible the study of the expression of the
25 genes of the HPV both at the level of transcription and translation, which is all the more interesting in that there does not exist at present a cell culture system capable of propagating the hepatitis B virus (HBV). Finally, cell infection by the recombinant adenovirus-HBV virus illustrates in a particularly advantageous manner the
30 methodological bases of a process for the production of a vaccine against a specific pathogenic agent (in this case the hepatitis B virus in the example considered).

Another nucleotide sequence of the genome of the hepatitis B virus of special interest is the S gene together with its pre-S2
35 region which codes for the HBs antigen and for a receptor of polymerized

human serum albumin (pHSA) (25), (26).

It will be obvious that it is possible to replace the S gene in the recombinant DNA by any other nucleotide sequence coding for a distinct protective antigen against another specific pathogenic agent, particularly when this distinct protective antigen is itself normally capable of being secreted by the cells transformed by the recombinant DNA. It will also be obvious that it is possible to replace the S gene and the pre-S2 region in the recombinant DNA by any other nucleotide sequence coding for a distinct protective antigen against another specific pathogenic agent, particularly when this distinct protective antigen is itself normally capable of being secreted by the cells transformed by the recombinant DNA. The nucleotide sequence coding for this distinct protective antigen may, moreover, possibly be inserted into the recombinant DNA in phase with another gene, for example the HBsAg antigen, provided that this other gene may be used as "locomotive" in order to promote the excretion of this distinct antigen as well, in particular in the form of a hybrid protein. As an example of distinct antigens capable of being thus produced (where appropriate in the form of a hybrid protein), mention may be made for example of the structural glycoproteins of the Epstein-Barr virus.

The first nucleotides of the nucleotide sequence coding for a specific polypeptide ("simple" or hybrid protein) are placed, in particular by construction as close as possible to this promoter, in particular to the "TATA box", characteristic of the promoter, it being nonetheless understood that the nucleotide sequence between the promoter and the initiator ATG of the nucleotide sequence coding for the said specific polypeptide should in general contain the triplets coding for the untranslated 5' end of the messenger RNA normally corresponding to the coding sequence and containing the sequences for binding to the ribosomes necessary for efficient translation. This untranslated 5' end of the messenger RNA may, moreover, be replaced by the untranslated 5' end of a messenger RNA distinct from that normally combined with a specific coding sequence. For example, in the case of the S gene, it is possible to replace the untranslated 5' end containing the pre-S gene or adjacent to this latter by the

untranslated 5' end of the messenger RNA of the T antigen of SV40. However, it has also been observed that when a DNA sequence containing the S and pre-S2 regions of the genome of the hepatitis B virus is used under the control of the strong E1A promoter, it is possible to obtain the expression of both the pre-S2 region and the S region. Any other untranslated 5' end of the messenger RNA may be used, provided that it is compatible with the other end similarly selected.

It is advantageous if the distance between the TATA box of the promoter and the initiation site of the messenger RNA is about 30 nucleotides.

The E1A promoter of the recombinant DNA according to the invention and also more generally the vector according to the invention making use of the most important parts of the genome of an adenovirus are preferably derived from an adenovirus belonging to the class C, such as has been defined by TOOZE. These adenoviruses have the known property of not being oncogenic. The sub-types Ad2 or Ad5 of this class of adenoviruses is characterized by a considerable transforming potency. The use of this latter type of recombinant DNA is thus particularly recommended when the desired expression product is intended for the production of protective antigens, in particular of active ingredients of vaccines. This is all the more true in the case in which whole and even infectious adenoviruses are used as active ingredients of live vaccines, in particular under the conditions which will be presented in more detail hereafter.

Naturally, the invention also relates to the cell lines, in particular of human or animal origin, which are transformed by recombinant DNAs such as defined above and which are made capable of synthesizing a polypeptide encoded in the nucleotide sequence (or the said nucleotide sequences) contained in these recombinant DNAs and placed under the direct control of the said promoter.

The invention also relates more particularly to the cell lines transformed by a recombinant vector conforming to the invention and, in addition, characterized in that the cells of these cell lines themselves contain a distinct sequence of nucleotides capable of complementing with the part of the genome of the adenovirus which the

above-mentioned vector lacks, the said distinct sequence being preferably incorporated into the genome of the cells of the said cell line.

Consequently, after being transformed by the recombinant vectors, the line 293 already mentioned above constitutes a preferred cell culture according to the invention. As a result of the complementation sequence which the cells of this line contain, considerable viral multiplication is observed within these cells and, consequently, so is the manifold expression of the sequence coding for the predetermined polypeptide. In the first case in which this coding sequence is the S gene, a considerable amount of HBsAg antigen is produced and excreted into the culture medium of these cells. In the second case in which the coding sequences are constituted by the S gene and the pre-S2 region of the hepatitis B virus, the recombinant adenovirus directs the synthesis of HBsAg particles possessing a receptor activity for pHSa in vitro. Injected into rabbits, this recombinant virus produces anti-HBsAg and anti-pHSa antibodies. This shows the possibility of using a recombinant adenovirus to express a gene both in vitro and in vivo.

The same vectors may also be used for the transformation of Vero cells under similar conditions.

The recombinant vectors according to the invention may also be used for the transformation of cells themselves not possessing the complementation sequence under the conditions which have been indicated above. It may then be necessary to perform a co-transformation of these latter types of cells, on the one hand, with the recombinant vector according to the invention and, on the other, with a non-defective adenovirus or a distinct recombinant DNA containing the sequences of adenovirus which the recombinant vector according to the invention lacks. It will certainly be possible to observe in this latter case the simultaneous production of HBsAg antigens (when the coding sequence contains the S gene) and of the adenovirus replicated and released by the cells thus transformed. The protective antigen formed may, however, where appropriate, be separated from the viral suspension, for example by placing the culture medium in contact with anti-

adenovirus antibodies, preferably immobilized on a solid support such as cross-linked agarose, marketed under the tradename SEPHAROSE. In any case, the presence of residual amounts of virus in the vaccinating preparation is only of relatively minor importance. In fact, the

5 adenovirus has only low pathogenic potency in man. It leads only to benign respiratory infections.

The low pathogenic potency of the adenoviruses, more particularly of those which belong to the class C of the human adenoviruses makes it possible to envisage the constitution of "live

10 vaccines". The latter may be constituted by infectious adenoviruses modified in their E3 region by the insertion of the recombinant DNA according to the invention in the inessential part of the adenovirus. In this respect, the fact should be emphasized that the human adenoviruses of group C have never been shown to be tumorigenic in

15 animals (3). These vectors or viruses will be of a quite special interest for the transformation of Vero cells, the non-tumorigenic character of which is now firmly established. As a result, they constitute a line of animal origin particularly favourable for the production of products for human use.

Additional characteristics of the invention will also become

20 apparent in the course of the description which follows of preferred constructions of vectors containing the recombinant DNA according to the invention and of the conditions under which these vectors can be used. For that purpose reference should be made to the drawings in

25 which:

- Fig. 1 presents schematically the relative positions of the different regions of the genome of an adenovirus of the sub-type Ad5, on the one hand and, on a larger scale, the E1A region of this genome;
- Fig. 2 is a from now on classic schema of the genome of the

30 hepatitis B virus;

- the Figs. 3 to 6 show the successive constructions which have led to the generation of a plasmid containing a first recombinant DNA in conformity with the invention (Fig. 6) and
- Fig. 7 presents schematically the construction of a "defective

35 recombinant virus" starting from the modified plasmid shown in Fig.

5 and the defective genomes of Ad5.

The following observations will first be made with respect to the figures before the generation of constructions of recombinant DNAs according to the invention are described.

5 In the Figs. 3 to 6, the parts shown with a fine line correspond to sequences of the plasmids pML2.

10 The numbers appearing in the Figs. 1 to 5 indicate the positions of the restriction sites in the viral sequences Ad5, SV40 and HBV. The numbering of the positions of the restriction sites of Ad5 and of SV450 are those of J. TOOZE (1), those of the HBV are those of P. THIOLLAIS et al. (11).

15 The crossings-out of the designations of the sites in the drawings point to the former presence of these sites in the corresponding parts of the DNAs which will be described hereafter. These sites have, however, been deleted, suppressed by repair of the cohesive ends of the open fragments or fragmented with the aid of corresponding restriction enzymes, or by any other agent as envisaged in the description which follows of the constructions which have been made.

20 1. Background to the principal elements of the structure and organisation of the genome of the adenovirus Ad5 (hereafter often simply referred to by the designation Ad5):

They result from the parts 1A and 1B of Fig. 1.

25 1A : The genome is a long molecule of linear double-stranded DNA of about 36,000 bp. The arrows indicate the position and the sense of the transcription of the early regions E1a, E1b, E2, E3 and E4. The major late promoter MP₁ and the transcription unit which is associated with it are also shown. The numberings in tens from 0 to 100 correspond to sizes expressed in % of the size of the total genome.

30 1B : The E1A region. Transcripts of this region all have identical 5'P ends (position 499) and 3'OH ends (position 1632). The first T of the TATA element of the promoter is situated at position 468. The restriction sites used in the construction of the plasmids are shown. The sizes are expressed as numbers of base pairs.

2. Origin of the fragments containing the S gene or the S gene and the pre-S2 region used in the constructions which follow.

1st case : Fragment of the genome of the hepatitis B virus containing the S gene.

It is derived from the genome of the hepatitis B virus (Fig. 2). It is recalled that the genome of the hepatitis B virus is a molecule of partially single-stranded circular DNA. Its length is about 3,200 bp. It is constituted by the pairing of two strands of unequal length called strands I(-) and S(+). The S gene represents the coding sequence of the major polypeptide of the viral envelope which bears the HBsAg. The DNA fragment used in the constructions below is the fragment XhoI₁₂₇-BglIII₁₉₈₄. The polyadenylation site of the messenger of the HBsAg was located at position 1916.

2nd case : Fragment of the genome of the hepatitis B virus containing the S gene and the pre-S2 region.

The DNA fragment used is the fragment MstII₃₁₆₁-BglIII₁₉₈₂ which codes both for the HBs antigen and for a receptor for polymerized human serum albumin (pHSA). The MstII site precedes the initiation codon of the pre-S2 region by 9 nucleotides. The BglIII site is situated 64 nucleotides downstream from the poly A addition signal of the S gene.

The subject of what follows is the construction and propagation of a recombinant adenovirus bearing the S gene. The procedure to obtain a recombinant adenovirus, conforming to the invention and possessing the S gene and the pre-S2 region is identical, it being understood that in the second case it is the DNA fragment MstII-BglIII which is inserted between the restriction sites HindIII and BamHI of the plasmid pK4, in place of the DNA fragment XhoI-BglIII referred to above.

In what follows, the recombinant adenovirus possessing the S gene will be designated as Ad5(X-B) and the recombinant adenovirus possessing the S gene and the pre-S2 region will be designated as Ad5 (M-B).

3. Construction of the plasmid pElA(TaqI) (Fig. 3).

The plasmid pElA(TaqI) contains the first 632 nucleotides

of the left-hand end of the genome of Ad5. This fragment was obtained by cleavage of the purified restriction fragment SaeI E (0 - 5.0%) of Ad5 by TaqI (Fig. 1). This fragment was inserted between the restriction sites EcoRI and ClaI of the plasmid pML2 (Fig. 3). The plasmid pML2 was opened by EcoRI and ClaI . The fragment of Ad5 was linked to the linearized plasmid at the TaqI end. The junction of the extremities TaqI - ClaI regenerates a ClaI restriction site. The EcoRI end of the recombinant was repaired with DNA-polymerase I of E.coli (Klenow fragment) and the plasmid was recircularised by means of the T4 ligase. The EcoRI site was thus reconstituted.

4. Production of the plasmid pAB1 (Fig. 4)

The plasmid pAB1 was constructed from the plasmid pElA(TaqI) so as to remove the coding part of the ElA region. This was performed by isolation of the PvuII - PvuII fragment (positions 452-623), cleavage of this fragment by the enzyme HaeIII (position 495), reinsertion of the PvuII_{452} - HaeIII_{495} fragment at the PvuII_{623} site of the plasmid pElA(TaqI). In other words, the HaeIII_{495} - Pvu_{623} fragment was deleted. The plasmid pAB1 contains a HindIII site close to the site for the initiation of transcription of the ElA region and a BamHI site (of pML2) situated further away. These two restriction sites may be used to clone foreign genes without there being genetic fusion.

5. Production of the plasmid pK4 (Fig. 5).

pAB1 was cut by means of HindIII and BamHI and the fragment containing the ElA promoter, derived from pAB1, was linked to the BglI - BamHI fragment (positions 5235-2533 of the genome of the SV40 virus) at its BamHI end, hereafter denominated A(SV40) containing the gene coding for the T and t antigens of the SV40 virus. After repair of the BglI and HindIII ends of the reconstituted plasmid by the Klenow fragment, it is recircularised by means of the T4 ligase. The construction present in the pK4 plasmid was tested by inducing the transient expression of the T gene. Introduced into HeLa cells by transfection according to the calcium phosphate procedure (19), the pK4 plasmid directs the synthesis of the T antigen of SV40 which was detected by immunofluorescence. About 1% of the transfected cells exhibit a marked fluorescence. The absence of fluorescence after

cellular transfection by a plasmid containing the SV40 fragment inserted in the incorrect orientation shows that the gene for the T and t antigens is correctly placed under the control of the ElA promoter of Ad5.

5 It is the HindIII site at position 5171 of the A (SV40) fragment which is then used to substitute the above-mentioned fragment containing the S gene for the major part of A(SV40).

6. Production of the plasmid pK4S (X-B) (Fig. 6).

10 The plasmid pK4 was digested by means of HindIII and BamHI. The XhoI-BglII fragment (positions 125 to 1982) of the genome of the hepatitis B virus (HBV) (Fig. 2) was inserted at the site and in place of the major part of A(SV40) between the restriction sites HindIII and BamHI of the pK4 plasmid, after repair of their ends by the DNA polymerase I of E.coli (Klenow fragment) (Fig. 3). The restriction sites XhoI, HindIII, BamHI and BglII are lost after ligation.

15 The HindIII site was situated 8 nucleotides upstream from the ATG initiation codon for the T and t antigens. The insertion of the S gene at this site thus makes it possible to conserve the 5' end of the early mRNA of the SV40 containing the "capping" site and the sequences base-pairing the messenger to the ribosomes. The DNA HBV fragment contains the coding sequence or S gene (position 155 to 833) of the major polypeptide of the viral envelope bearing HBsAg as well as the sequence situated at the 3' of the S gene and which includes the polyadenylation site of the messenger RNA of the HbsAg at position 1916 (20, 21, 22). Two plasmids pK4S⁺ and pK4S⁻ bearing the HBV fragment inserted in both senses were isolated. 293 cells were transfected with these two plasmids and the synthesis of HBsAg was assayed in the cell supernatant 3 days after transfection. Only the plasmid pK4S⁺, which possesses the ElA promoter at the 5' end of the S gene, is capable of directing the synthesis of HBsAg. This shows that the expression of the S gene is indeed under the control of the ElA promoter of Ad5. Finally, a non-methylated ClaI restriction site in E.coli was introduced into the plasmid pK4S⁺ (X-B) at the NruI site of the sequence pML₂. The site is necessary for the construction of the recombinant virus.

35 Finally, it is the fragment delimited by PstI and ClaI ends,

obtained from pK4S⁺ (X-B), which was used for the production of a "defective recombinant virus" in conformity with the invention.

7. Construction of the defective recombinant virus (Fig. 7).

3 Micrograms of the restriction fragment PstI-ClaI purified from the plasmid pK4S⁺ (X-B) were ligated to 20 micrograms of the restriction fragment ClaI (2.6% - 100%) of the Ad5 purified by ultracentrifugation in a sucrose gradient to obtain the Ad5 "recombinant defective virus".

8. Propagation of the recombinant virus.

10 The 293 cells were cultivated in dishes 6 cm in diameter. 4 hours before transfection, the culture supernatant was replaced by medium. 5 dishes of 293 cells at 70% confluence were then transfected with the ligation mixture according to the calcium phosphate procedure, then incubated for 4 hours at 37°C. After adsorption, the cells 15 contained in each dish were washed with 2 ml of TS buffer (NaCl 8000.0 mg/l, KCl 380.0 mg/l, Na₂HPO₄ 100.0 mg/l, CaCl₂ 100.0 mg/l, MgCl₂, 6H₂O 100.0 mg/l, Tris 3000.0 mg/l pH 7.4), treated with 400 microliters of a TS solution containing 20% of glycerol for 1 minute at room temperature, washed twice with 2 ml of TS buffer, then covered again 20 with 4 ml of MEM medium containing 1% agar, 1% fetal calf serum. On days 4 and 7, the cells were recovered with 4 ml of the nutritive mixture. On day 10, the cells were stained with 4 ml of nutritive medium supplemented with 0.01% of neutral Red. The plaques were observed on day 11. The viruses were resuspended in 1 ml of TS and amplified in 25 293 cells. The presence of HBsAg in the culture medium was tested by RIA (Austria II, ABBOTT laboratories). After amplification, the presence of HBV sequences in the recombinant was tested by hybridization. Five plaques were analysed. Only one contained a HBsAg⁺ recombinant virus. This clone Ad5(X-B) as well as another clone were positive for the 30 detection of HBV sequences. The size of the recombinant viral genome exceeded that of the wild-type virus by 2100 bp. No deletion could be detected by analysis of the restriction fragments of the recombinant genome. Furthermore, this analysis showed that the sequences of the pMI2 situated between the PstI restriction site and the Ad5 sequence 35 were correctly excised during the propagation of the recombinant genome

in the 293 line.

9. The synthesis of HBsAg directed by the vector Ad5(X-B).

293 cells and Vero cells were infected with the virus Ad5(X-B). The levels of expression of HBsAg synthesized are shown in Table I. Samples of the cell supernatant were taken 3 days after the infection and the HBsAg was assayed by means of radioimmunological assay ("radio-immunoassay" : RIA). The results show that the vector Ad5 (X-B) is capable of directing the synthesis of HBsAg in these two cell lines, and the excretion of HBsAg by the cell lines into their respective culture medium.

The HBsAg synthesized was purified by ultracentrifugation in CsCl. It has a density of 1.20. Typical particles of 22 nm were observed by means of electron microscopy.

10. Synthesis of HBsAg directed by the vectors Ad5 (M-B).

The levels of expression of the HBsAg synthesized after infection of 293 cells and Vero cells with Ad5 (M-B) are shown in Table I.

The kinetics of the extra- and intra-cellular distribution of HBsAg starting from Vero cells infected with Ad5(M-B) indicated that the synthesis of HBsAg began 3 hours after infection and could be detected in the medium after 8 hours. The infection by the recombinant virus Ad5(M-B) led to an accumulation of HBsAg of 0.5 to 1 $\mu\text{g}/10^6$ cells in the medium after 120 hours. Repeated experiments have shown that the recombinant virus containing the pre-S2 region synthesizes larger quantities of HBsAg than the recombinant virus containing only the S gene. The HBsAg purified from the culture medium of cells infected with the recombinant adenovirus Ad5(M-B) consisted of a homogeneous population of particles having a mean diameter of 22 nm. The density after centrifugation in CsCl was 1.21.

11. Receptor activity for pHSA of the HBsAg particles derived from Vero cells.

The HBsAg particles produced in the Vero cells were tested by the hemagglutination procedure using sheep red blood cells covered with pHSA and by radioimmunological assay (RIA) in order to detect the presence of a receptor activity for pHSA. A binding activity for

the pHSA was detected, but not for polymerized bovine albumin (Table II). Such activity was not detected with the Vero cells infected with the recombinant adenovirus Ad5(X-B) containing only the S gene.

12. In vivo activity of the recombinant virus.

5 Rabbits were inoculated intravenously with highly purified preparations of the recombinant adenovirus Ad5(M-B) and of the wild-type adenovirus. Although the HBsAg antigen could not be detected in their serum, 5 out of 8 of the rabbits inoculated with the recombinant virus showed the appearance of an anti-HBs titer varying from 20 to 10 270 mIU/ml after 15 days (Table III). No anti-HBs antibodies were detected in the rabbits which were injected with the wild-type adenovirus. After a second intravenous inoculation performed 4 weeks after the first, a second peak was observed in the anti-HBs response which attained 440 mIU/ml in the case of one of the animals. 4 weeks 15 after the second injection, anti-HBs titers varying from 6 mIU/ml to 360 mIU/ml were found. Earlier studies had shown that the minimal level of anti-HBs which is still protective against the HBV is 10 mIU/ml for man. The presence of anti-pHSA antibodies was investigated in the rabbits inoculated for the purpose of determining their relationship 20 with the neutralization of the HBV. These antibodies, detected by the inhibition of hemagglutination, were found in 5 animals out of 5 showing a positive anti-HBs response (Table IV).

 The recombinant adenovirus Ad5(M-B) thus directs the in vivo synthesis of HBsAg particles which have the property of a receptor for 25 polymerized human serum albumin.

 The invention thus provides a methodological basis for the production of a vaccine against hepatitis B (or against other types of infections) in cell cultures.

 The use of the adenovirus type 5 as vector is important in 30 two ways. On the one hand, this virus is a virus whose pathogenic potency in man is low. It only leads to benign respiratory infections. On the other hand, this serotype which belongs to class C of the human adenoviruses is not tumorigenic in animals. Furthermore, the level of production of HBsAg obtained in Vero cells (about 1 microgram/10⁶/ 35 per infectious cycle) is a priori sufficient for industrial

exploitation. This cell line is not tumorigenic and for this reason it constitutes the most favourable line of animal origin for the production of a product for human use.

5 It will also be obvious that the 293 cells which were mentioned above may be replaced by any other cells of higher eukaryotes which can be infected with adenoviruses or are capable of recognizing the E1A promoter of the adenoviruses, these cells having been modified by the incorporation beforehand into their own genomes of a sequence containing the parts of the defective recombinant virus according to 10 the invention lacking from the genome of an adenovirus, in particular under the control of a strong promoter recognized by these cells, for example a promoter of thymidine kinase or a promoter of the SV40 virus. The original sequence of the adenovirus, then integrated into the genome of these higher eukaryotic cells, will then complement the defective 15 viruses conforming to the invention under conditions similar to those permitted by the 293 cells. These methods can be used with Vero cells to particular advantage.

The adenovirus Ad5 used was deposited on 3 August 1984 under No. I-322 with the C.N.C.M. ("Collection Nationale de Cultures de Micro- 20 organismes de l'INSTITUT PASTEUR de Paris.

The line 293 was deposited with the C.N.C.M. on 3 August 1984 under No. I-323.

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TABLE IProduction of extracellular HBsAg as a function of time

Day	Ad5 (X-B)- infected		Ad5 (M-B) - infected	
	Vero cells	293 cells	Vero cells	293 cells
1	13	15	106	35
2	17	90	N.D.	N.D.
3	45	110	349	56
4	110	100	668	60
5	320	130	1 057	63
6	470	120	1 153	N.D.
7	550	120	900	N.D.

The cumulative amounts of HBsAg (in ng) produced after infection of 293 cells and Vero cells, either by Ad5(X-B), or by Ad5(M-B) are indicated.

TABLE II

Receptor activity for pHSA of the HBsAg particles from Vero cells

Cell culture supernatant						Human serum			
Ad5(X-B)		Ad5(M-B)		Uninfected control		HBsAg+ HBeAg+		Témoin sain	
RIA	HA	RIA	HA	RIA	HA	RIA	HA	RIA	HA
pHSA	143	-	7 512 64	119	-	10 472 128	65	-	-
pBSA	58	ND	85 ND	77	ND	145 ND	81	ND	-

The results are expressed in cpm in the case of the radio-immunological assay on a solid phase and as hemagglutination titer (HA).

ND. not determined.

pHSA : polymerized human serum albumin

pBSA : polymerized bovine serum albumin

cpm : counts per minute.

TABLE III

Anti-HBs response (mIU ml⁻¹) in the inoculated rabbits

Weeks	Rabbits									
	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
1	0	0	2	0	0	30	8	6	12	20
2	2	0	2	0	2	15	20	270	70	85
3	0	2	0	0	5	18	13	48	15	11
4	1	0	0	0	2	21	15	27	17	7
5	0	0	2	0	2	99	52	440	146	136
6	0	0	0	0	0	78	26	401	151	148
7	0	0	0	0	0	20	27	325	58	42
8	0	0	0	0	0	20	40	367	35	6

An intravenous injection of 10^9 pfu of purified wild-type adenovirus Ad5 was given to rabbits (rabbits 1 and 2) and of 10^9 pfu of purified recombinant adenovirus Ad5 (M-B) was given to rabbits (rabbits 3 to 10) immediately after the blood sample had been taken at week 0 and at week 4. The amount of anti-HBs antibody was measured by using the Abbott AUSAB RIA system and expressed in international units (3.5 RIA equivalent to 1 mIU).

(pfu = plaque forming unit).

TABLE IV

Immunogenic response of the anti-pHSA receptor (reciprocal of the titer)

5	Weeks	Rabbits				
		6	7	8	9	10
10	0	0	0	0	0	0
	1	16	2	2	2	2
	2	4	16	16	16	16
	3	4	4	16	8	8
	4	2	2	4	4	4
15	5	16	32	32	32	32
	6	16	32	32	16	32
	7	8	8	32	8	16
	8	4	4	8	4	8

Rabbits were infected intravenously with 10^9 of purified recombinant adenovirus Ad5(M-B) at weeks 0 and 4. The animals were identified by the same numbers as in the Table III.

The anti-pHSA receptor activity was expressed as the reciprocal of the highest serum titer capable of giving rise to 100% inhibition of hemagglutination. The HBsAg particles possessing a receptor hemagglutination titer for pHSA of 1 : 128 were mixed with an equal volume of dilution series of serum inhibitors.

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CLAIMS

1. Recombinant vector containing the early promoter of the E1A region of the genome of an adenovirus for the transformation of eucaryotic, particularly human or animal cell lines, selected among those which are infectable by adenoviruses or whose endogenous polymerases are liable of recognizing the promoters of adenoviruses, said vector being further modified by a nucleic acid insertion containing a nucleotide sequence coding for a polypeptide sequence whose expression in said eucaryotic cell lines is sought, characterized in that said insertion sequence is substituted for the E1A genes normally placed under the direct control of said early promoter of the E1A region, said insertion sequence being itself placed under the direct control of said promoter, and in that it comprises, downstream of said nucleic acid insertion, all of the essential sequences of an adenovirus genome which are necessary for the replication of the corresponding adenovirus and which are normally located downstream of the genes normally under the direct control of the E1A early promoter in said genome, and in that said nucleotide sequence coding for the polypeptide sequence whose expression is sought is heterologous with respect to the genes of said adenovirus.

2. Recombinant vector according to claim 1, characterized in that the genome of the adenovirus with which it is associated is defective, in that it is deprived of the anterior part of the E1A region of the viral genome, particularly of its 0-2,6 % fragment.

3. Recombinant vector according to claim 1, characterized in that the recombinant DNA formed by said early promoter of the E1A region and the



insertion nucleic sequence is incorporated in the non-essential region of an adenovirus, particularly in the non-essential part of the E3 region of said adenovirus.

4. Recombinant vector according to claim 1, characterized in that it consists of an infectious adenovirus comprising the recombinant DNA formed by the early promoter of the E1A region and the nucleic sequence insertion, incorporated in one of its non-essential regions, particularly in the non-essential part of the E3 region.

5. Recombinant vector according to any one of claims 1 to 4, characterized in that its promoter originates from an adenovirus of category C, belonging to sub-type Ad2 or Ad5.

6. Recombinant vector according to any one of claims 1 to 5, characterized in that the nucleotide sequence contained in the aforesaid nucleic acid insertion codes for a protein which, when it is expressed in a natural cellular host under the control of its natural promoter, is excreted into the culture medium of this natural cellular host.

7. Recombinant vector according to any one of claims 1 to 6, characterized in that the aforesaid nucleotide sequence codes for a protective antigen against a given pathogenic agent, particularly for the HBsAg antigen.

8. Recombinant vector according to claim 7, characterized in that said nucleic acid sequence comprises the S gene and the pre-S2 region of the genome of hepatitis B.

9. A cell line of human or animal origin, characterized in that it is transformed by a recombinant vector according to any one of claims 1 to 8, and in that it synthesizes the polypeptide coded by the aforesaid nucleotide sequence.



10. The cell line according to claim 9 considered in combination with claim 1 or claim 2 or both at once, and, as the case may be, with any one of claims 5 to 8, characterized in that it itself contains a distinct sequence of nucleotides able to complement the part of the genome of the adenovirus which the aforesaid vector is deprived of, the said distinct sequence preferably being incorporated into the genome of the cells of said cell line.

11. A process of production of a determined polypeptide including the transformation by a recombinant vector containing a nucleotide sequence coding for said polypeptide of the cells of a eucaryotic, particularly animal of human cell line, chosen among those which are infectable by the adenoviruses or whose endogenous polymerases are able to recognize the promoters of the adenoviruses, characterized by the fact that the transforming DNA is in accordance with any one of claims 1 to 8 and in that the expression products, including said specified polypeptide of these cells are recovered.

12. The process according to claim 11, characterized in that the recombinant vector is in accordance with any one of claims 5 to 8.

13. The process according to claim 11, characterized by the fact that the aforesaid transformed eucaryotic cells are in accordance with claim 10.

14. The process according to claim 11, characterized by the fact that the aforesaid transformed eucaryotic cells are simultaneously transformed with the corresponding infectious adenovirus.

15. The process according to any one of claims 11 to 14, characterized in that the specific



nucleotide sequence is in accordance with that defined in claim 7.

16. A process of production of a polypeptide possessing the immunogenic properties characteristic of the virus of hepatitis B including the transformation of a line of eucaryotic, particularly animal or human cells, chosen from among those which are infectable by adenoviruses of whose endogenous polymerases are able to recognize the adenovirus promoters, by a recombinant vector containing an insert itself containing a nucleotide sequence coding for an immunogenic polypeptide characteristic of the virus of hepatitis B, characterized in that the aforesaid nucleotide sequence is substituted for the E1A genes normally placed under the direct control of the early promoter of the E1A region of the genome of an adenovirus, said insertion sequence being itself placed under the direct control of said promoter and said recombinant vector further containing, downstream of said nucleic acid insertion, all of the sequences of an adenovirus genome which are essential and necessary for the replication of the corresponding adenovirus and which are normally located downstream of the genes normally under the direct control of the early E1A promoter in said genome.

17. The process according to claim 16, characterized in that the recombinant vector consists of a defective adenovirus which can be replicated in said cell lines, said adenovirus being deprived of the anterior part of the E1A region of the viral genome, particularly of its 0-2,6 % fragment, and in that one recovers in the culture medium the expression products of the eucaryotic cell lines transformed by the recombinant DNA, including the polypeptide coded by the aforesaid nucleotide sequence and synthesized by said cell lines.



18. The process according to claims 16 to 17, characterized in that the promoter of the recombinant DNA originates from an adenovirus of category C, belonging to sub-type Ad2 or Ad5.

19. The process according to any one of claims 16 to 18, characterized in that the recombinant DNA formed by said early promoter and said insertion sequence is incorporated into a non-essential region of the adenovirus, particularly into the non-essential part of the E3 region of this adenovirus.

20. The process according to any one of claims 16 to 19, characterized in that the eucaryotic cell line itself contains a distinct sequence of nucleotides able to complement the part of the genome of the adenovirus of which the aforesaid vector is deprived, the said sequence preferably being incorporated into the genome of the cells of said cell line.

21. The process according to any one of claims 16 to 20, characterized in that the recombinant DNA includes a nucleotide sequence corresponding to the S gene of the genome of the virus of hepatitis B.

22. The process according to any one of claims 16 to 20, characterized in that the recombinant DNA includes the sequence corresponding to the S gene and the pre-S2 region of the genome of the virus of hepatitis B.

23. Vaccine whose active principle consists of an adenovirus which is infectious and can be replicated and which comprises, incorporated into one of its non-essential regions, particularly the non-essential part of its E3 region, a DNA recombinant formed between the early promoter of the E1A region and a nucleic sequence insertion coding for a protective antigen against the pathogenic agent selected, and particularly against the hepatitis B.



virus, said nucleic sequence insertion being substituted for the E1A genes normally under the control of said promoter.