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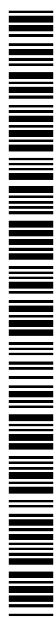

(54) Title: MINI-ADENOVIRAL VECTOR SYSTEM FOR VACCINATION

(57) Abstract: The principle of this invention is to produce mini-Ad vectors using packaging-attenuated and replication-defective helper Ad and an E1-complementing Ad helper cell line. Since the essential *cis*-acting elements for Ad DNA replication and packaging are located at the ends of the viral genome (ITRs plus the packaging signal, less than 1 kb), the backbone of the mini-Ad vectors is trimmed down to contain only the essential *cis*-elements. The remainder of the Ad genome is to be replaced by non-viral DNA for gene transfer, retention, and expression. The capacity of the mini-Ad vectors may be up to 36 kb. The viral proteins required for DNA replication and encapsidation of the mini-Ad vectors are designed to be provided *in trans* from the helper Ad (*trans*)-complementation) and the helper cell line. In order to generate relatively pure preparations of the mini-Ad virions, packaging of the helper Ad genome is controlled by selective attenuation of the packaging signal while that of the mini-Ad vector genome proceeds normally. In the absence of mini-Ad vector genome, the helper Ad does replicate and is propagated in an inefficient manner. In this system, the Ad helper cell line is necessary for the E1-complementation of the helper virus and may also provide other functions for packaging attenuation of the helper viral genome and enhancement of mini-Ad vector replication. If the preparation of mini-Ad vectors is not sufficiently pure, biochemical or other physical methods will be utilized to achieve further purification.

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MINI-ADENOVIRAL VECTOR SYSTEM FOR VACCINATION

FIELD OF THE INVENTION

This invention is related to adenoviral (Ad) vectors and their applications in the field of genetic medicine, including gene transfer, gene therapy, and gene vaccination. More specifically, this invention is related to the Ad vectors that carry the minimal *cis*-element of the Ad genome (mini-Ad vector) and are capable of delivering transgenes and/or heterologous DNA up to 36 kb. The generation and propagation of the mini-Ad vectors require *trans*-complementation of a packaging-attenuated and replication-defective helper Ad (helper) in an Ad helper cell line.

BACKGROUND OF THE INVENTION

An important issue in the development of genetic medicine is the development of ideal gene delivery systems. The ideal system of gene delivery must possess several properties that are currently unavailable in a single gene therapy vector. The ideal vector must retain adequate capacity to accommodate large or multiple transgenes including regulatory elements and be amenable to simple manipulation and scale-up for manufacturing. Such a vector must also be be safe and demonstrate low toxicity as well as demonstrate highly efficient and selective delivery of transgenes into target cells or tissues. Finally, such a vector must be capable of supporting appropriate retention, expression, and regulation of the transgenes in target cells. The present invention encompasses a novel design of a high-capacity and highly-efficient Ad vector system and is focused on resolving the issues and concerns of those skilled in the art regarding an ideal gene delivery system.

Adenoviruses (Ad) consist of nonenveloped icosahedral (20 facets and 12 vertices) protein capsids with a diameter of 60-90 nm and inner DNA/protein cores (1). The outer capsid is composed of 252 capsomers arranged geometrically to form 240 hexons (12 hexons per facet) and 12 penton bases; the latter are located at each vertex from which protrude the antennalike fibers. This structure is responsible for attachment of Ad to cells during infection. Wild-type Ad contain 87% protein and 13% DNA and have a density of 1.34 g/ml in CsCl.

The double-stranded linear DNA genome of Ad is approximately 36 kb, and is conventionally divided into 100 map units (mu). Each end of the viral genome has a 100-150 bp

repeated DNA sequence, called the inverted terminal repeats (ITR). The left end (194-385 bp) contains the signal for encapsidation (packaging signal). Both of the ITRs and the packaging signal are the *cis*-acting elements necessary for viral DNA replication and packaging (2, 3).

A simplified map of the adenovirus type 5 (Ad5) genome with a few key landmarks is diagrammed in Figure 1 (4, 5). The early (E) and late (L) regions of the genome that contain different transcription units are divided according to the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome as well as a few cellular genes (6). The expression of the E2 region (E2A and E2B) leads to the synthesis of the proteins needed for viral DNA replication (7). The proteins from the E3 region prevent cytolysis by cytotoxic T cells and tumor necrosis factor (8). The E4 proteins are involved in DNA replication, late gene expression and splicing, and host cell shut off (9). The products of the late genes, including the majority of the viral capsid proteins, are expressed after processing of a 20-kb primary transcript driven by the major late promoter (MLP) (10). The MLP (located at 16.8 mu) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence, which makes them preferred over the cellular mRNA for translation.

The use of Ad as vectors for expression of heterologous genes began soon after the observation of hybrids between Ad and simian virus 40 (SV40) during the 1960s. Since then, Ad vectors have gradually developed into one of the major viral vectors in the field of gene therapy, mainly for the following reasons: (a) Ad have been widely studied and well characterized as a model system for eukaryotic gene regulation, which served as a solid base for vector development; (b) the vectors are easy to generate and manipulate; (c) Ad exhibit a broad host range *in vitro* and *in vivo* with high infectivity, including non-dividing cells; (d) Ad are relatively stable and may be obtained in high titers, e.g., 10^{10}-10^{12} plaque-forming unit (PFU)/ml; (e) the life cycle of Ad does not require integration into the host cell genome, and the foreign genes delivered by Ad vectors are expressed episomally, thus having low genotoxicity if applied *in vivo*; (f) Side effects have not been reported following vaccination of U. S. recruits with wild-type Ad, demonstrating their safety for *in vivo* gene transfer. Furthermore, Ad vectors have been successfully used in eukaryotic gene expression (11, 12), vaccine development (13, 14), and gene transfer in animal models (4, 15, 16). Experimental routes for administrating recombinant Ad to different tissues *in vivo* were intratracheal instillation (17), muscle injection (18), peripheral

intravenous injection (19), and stereotactic inoculation to brain (20). The first trial of Ad-mediated gene therapy in human was the transfer of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to lung (21).

The size limit on DNA that can be packaged into Ad virions is quite close to the size of the wild-type Ad genome. The largest described Ad-SV40 hybrids are approximately 2% larger than Ad itself. Thus, insertion of large fragments of heterologous DNA into Ad requires replacement of viral sequences. In order to make viable recombinant Ad that can carry heterologous DNA fragments, the function of the replaced viral DNA has to be either dispensable or compensated through a *trans*-acting source. The *trans*-acting function is actually a function of a helper, which may take the form of either helper virus or a helper cell, or both. Currently, there are two types of Ad vector systems based on different types of helper function.

The helper virus-dependent Ad were the early and original type of infectious recombinant Ad, which was gradually developed into a vector system for delivering heterologous genes, since the discovery of Ad-SV40 hybrid viruses. This approach has a strong shortcoming, because of helper virus contamination to the recombinant virus. Its development has been further slowed by a recently developed approach including generation of helper virus-independent and replication-defective vectors.

Helper virus-independent Ad vectors became possible through the development of the first Ad helper cell line, 293, which was derived from human embryo kidney cells by transformation with Ad5 DNA fragments and constitutively expresses E1 proteins (22) (Fig. 2). Three major approaches have been used for generation of replication-defective and helper virus-independent recombinant Ad. The first was the Stow method (23), which is an *in vitro* recombination approach, using the modified E1 region with the most left end of the Ad genome to ligate with the isolated Xba I large fragment of the Ad genome (3.7-100 mu) of *dl*309, and transfecting the artificial adenoviral genome into 293 cells for production of new viruses (Fig. 3A). The second is an approach of *in vivo* recombination, which mixes the modified left arm of the Ad genome with a left-endless Ad genome overlapping with the left arm and co-transfecting the DNA into 293 cells for generating Ad through homologous recombination (24). The third method is an approach of plasmid recombination (25). This method generates recombinant Ad through co-transfecting shuttle and recombinant plasmids into 293 cells (Fig. 3B).

The E3 region is dispensable from the Ad genome (26), and the first generation Ad vectors, carry foreign DNA in either E1 or E3 or both regions (5). In nature, Ad can package total DNA approximately 105% of the wild-type genome (27), which provides the capacity for an additional 2 kb of DNA. Combined with the approximately 6.5 kb of DNA that is replaceable in
5 the E1 and E3 regions, the maximum capacity of the current Ad vector is under 8.5 kb, which occupies approximately 15% of the total length of the vector. Approximately 78% of the wild-type Ad genome remains in the vector backbone. Since the replication deficiency rendered by the E1 deletion is not complete (28), leakage of viral gene expression and replication may occur with the first generation Ad vectors at high multiplicities of infection (moi). Leakage represents a
10 source of vector-borne cytotoxicity in target cells and is responsible for induction of inflammatory and immune responses to the vector-infected target cells *in vivo* (29). These factors predispose a non-sustained nature of transgene expression in current Ad-mediated gene therapy.

The second generation of Ad vectors were designed to suppress the leakage of expression of the remaining viral genes by ablation of E2A through the introduction of a temperature-
15 sensitive (*ts*) mutant of the E2A gene (30). Mice that received a second generation Ad vector comprising the b-gal gene (*E. coli LacZ* gene encoding b-galactosidase) were reported to express the b-gal transgene for a longer period of time than those mice that received a first generation b-gal adenovirus. In addition, the inflammatory responses were shown to be delayed and of lower intensity in the mouse livers infected with second generation Ad vectors. However, the problems
20 associated with the first generation of Ad vectors were not completely resolved.

Approaches toward the development of adenoviral vectors into which large amounts of heterologous DNA may be introduced include the development of cell lines that are able to supply multiple adenoviral proteins in trans. An example is a 293 cell line expressing adenoviral E2A proteins that was generated by Dr. Heshan Zhou at Baylor College of Medicine in Houston.
25 Several groups have also developed cell lines with E1 plus E4 genes (31). Because the E2A and E4 gene products are toxic to cells, these genes were all transduced into 293 cells under inducible promoters. However, the artificial control of these genes has not been adequately matched to the tight temporal control program of Ad for gene expression and DNA replication required for successful viral replication. This inevitably results in lower titer output of the vectors from those
30 cell lines. Although further displacement of the viral genes from the adenoviral vector into the helper cell lines resulted in improved Ad helper cell lines, these modifications only increased the

gene-carrying capacity up to 11 kb. More than two thirds of the wild-type Ad genome remains in the vectors. So far, there have been no reports that the so-called second generation Ad vectors demonstrate sufficient improvements to resolve the above-described difficulties associated with currently available Ad vectors.

5 Recently, the rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector was reported (32). This vector system was designed to replace an essential region of the viral genome (L1, L2, VAI, VAII, and pTP) with an indicator gene. In the presence of wild-type adenovirus as a helper, the vector DNA was packaged and propagated as infectious viral particles. The recombinant virus was shown to be partially purified from the helper virus 10 by CsCl equilibrium density-gradient centrifugation. This represents an alternative approach to use of Ad helper cell lines. However, this system was not shown to reduce or eliminate co-generation of helper virus. The contamination of wild-type virus in the preparation of the recombinant Ad vectors remains problematic.

To address the problems associated with the current Ad vector systems and based on 15 understanding of the structure and function of both the ITR and packaging signal of the virus, the present invention provides a unique and more efficient system than others currently available. The design of the present invention is primarily focused on maximal removal of viral sequences from the Ad vector. The vector thus produced has minimal amount of viral *cis*-acting elements that includes the ITR sequences and a functional packaging signal. The present invention also 20 provides for decreased viral protein-mediated cytotoxicity, as well as decreased induction of inflammatory and immune responses due to the adenoviral vector. The present invention, designated the mini-Ad vector, is used to deliver transgenes and regulatory elements for transgene retention and expression. A technical difficulty in producing a mini-Ad vector is the design of an efficient helper function. The present invention includes a packaging- attenuated 25 or deficient Ad helper virus and assistance from a 293 cell like helper cell line. The helper virus is prevented from contaminating the mini-Ad vectors through selectively disabling packaging of the helper virus. The present invention includes a set of designs for manipulating the packaging signal in the helper virus. Other novel aspects of the present invention include development of novel Ad helper cell lines and novel mechanisms within the mini-Ad vectors for transgene 30 retention, expression, and regulation.

The development of the mini-Ad vector system has been based on the following observations:

1. The origins of Ad DNA replication are localized in the ITR sequences of the viral genome (33).

5 2. DNA replication of Ad, which is uniquely primed by the covalently bound terminal protein at each 5' end (34), directs asymmetric replication of a unit-length linear molecule (35). Thus, linear unit-length molecules are the substrate for packaging (36).

3. The packaging signal for viral encapsidation is localized between 194-385 bp (0.5-1.1 mu) at the left end of the viral genome (37). This signal mimics the protein recognition site in

10 l phage DNA where a specific sequence close to the left end but outside the cohesive end sequence mediates the binding to proteins that are required to insert l DNA into the head structure (38).

4. Studies using Ad E1-substitution vectors demonstrated that a fragment of the left end of the viral genome as short as 450 bp (0-1.25 mu) was sufficient to allow recombinant virus to

15 be packaged in 293 cells (11). In cell line W162 (39), constitutive expression of E4 proteins supported propagation of E4 deletion (92-99 mu) mutants.

5. Ad mutants having deletions in other regions throughout the viral genome were rescued by helper viruses that provided the deleted gene products *in trans* (40).

6. Ad-SV40 hybrids occurred in nature in the presence of wild-type Ad as helper viruses.

20 The hybrids have the majority of the Ad genome (up to 35 kb in length except the ends) substituted with the SV40 genome (41).

7. The packaging-attenuated helper virus has been shown to be useful in developing a Sindbis RNA viral vector system (42).

8. Total genome replacement for generation of recombinant viruses has been successfully

25 utilized in the construction of retroviral vectors (43).

The process of the Ad genomic replication, transcription, and encapsidation is tightly controlled in a precise temporal manner. As long as *trans*-complementation from the helper viruses or from both the helper virus and the helper cell line to the mini-Ad vectors is thoroughly designed without any missing elements, viral propagation should proceed in a proper manner.

30 Packaging selection favoring the mini-Ad vectors allows for preferential amplification of the mini-Ad vector after activation of the helper Ad packaging-attenuation mechanism.

Beyond the advantages already discussed, Ad have the potential to accommodate foreign DNA up to 36 kb. No other viral vector thus far characterized has as many benefits as Ad for supporting construction of the mini-viral vector system. The present invention provides the means with which to more efficiently utilize the Ad vector system.

5 Retroviruses were developed as gene transfer vectors relatively earlier than other viruses, and were first used successfully for gene marking and transducing the cDNA of adenosine deaminase (ADA) into human lymphocytes. However, the genotoxicity caused by the viral integration into the host genome, its reversion back to replication-competent retrovirus (RCR), its relative instability, and the dependence of its infectivity on target cell receptors and 10 proliferation limit its wide use in *in vivo* gene delivery (43). The maximum gene-carrying capacity of retroviral vectors is under 10 kb, which eliminates them from consideration as candidates for the construction of the large gene delivery system.

Adeno-associated virus (AAV) has recently been intoduced as a gene transfer system with potential applications in gene therapy. Wild-type AAV demonstrates high-level infectivity, broad 15 host range and specificity in integrating into the host cell genome (44). However, experimental data has shown that recombinant AAV are generated at low titer and eventually lose the specificity of integration (45). Its maximum gene-carrying capacity of under 5 kb also makes it inadequate for the objective of this invention.

Herpes simplex virus type-1 (HSV-1) is attractive as a vector system for use in the 20 nervous system because of its neurotropic property (46). The HSV-1 genome has over 70 genes located along a 150-kb DNA molecule, but the maximun amount of foreign DNA that may be carried by the virus has been reported to be 7 kb. A complementing cell line (E5 cells) is available for propagation of replication-defective HSV-1 and allows for about 5 kb of foreign DNA to substitute for the ICP4 gene (47). Because of the complexity of the HSV genome, it is 25 clear that a much greater understanding of the interactions of HSV with host cells is required before a suitable HSV vector system may be realized. The difficulties associated with the development of an appropriate HSV vector backbone and resolution of issues related to gene expression during latency have been investigated but not yet solved (48).

Vaccinia virus, of the poxvirus family, has been developed as an expression vector (49). 30 The vaccinia genome is among the most complex of all animal viruses, comprising approximately 200 discrete protein-coding regions along a nearly 200-kb DNA molecule. It has

been shown that approximately 25 kb of foreign DNA could be inserted into the viral genome and that the recombinant genome was still packaged into the virion (50). However, because of the life cycle of vaccinia virus limitations are inherent in the infectious vectors derived from it. For example, all infectious vectors are cytocidal. Unless this limitation is overcome, vaccinia virus is not suitable for gene therapy *in vivo*.

Other viral vectors are available, but they are neither well developed nor suitable for fulfilling the goal of this invention. This brief review of the viruses and their vectors clearly indicates that Ad is a good choice for the construction of the mini-Ad vector system.

When the entire field of genetic medicine is surveyed, gene delivery vectors with large-capacity, low toxicity, and controllable transgene retention and expression are an immediate need for overcoming the current limitations in gene therapy. To address this demand, none of the vectors currently available satisfies this challenge (51) and a new system must be developed. The present invention provides for the current demand of the field and meets all or at least a large portion of the demand.

SUMMARY OF THE INVENTION

The principle of this invention is to produce mini-Ad vectors using packaging-attenuated and replication-defective helper Ad and an E1-complementing Ad helper cell line. Since the essential *cis*-acting elements for Ad DNA replication and packaging are located at the ends of the viral genome (ITRs plus the packaging signal, less than 1 kb), the backbone of the mini-Ad vectors is trimmed down to contain only the essential *cis*-elements. The remainder of the Ad genome is to be replaced by non-viral DNA for gene transfer, retention, and expression. The capacity of the mini-Ad vectors may be up to 36 kb. The viral proteins required for DNA replication and encapsidation of the mini-Ad vectors are designed to be provided *in trans* from the helper Ad (*trans*-complementation) and the helper cell line. In order to generate relatively pure preparations of the mini-Ad virions, packaging of the helper Ad genome is controlled by selective attenuation of the packaging signal while that of the mini-Ad vector genome proceeds normally. In the absence of mini-Ad vector genome, the helper Ad does replicate and is propagated in an inefficient manner. In this system, the Ad helper cell line is necessary for the E1-complementation of the helper virus and may also provide other functions for packaging attenuation of the helper viral genome and enhancement of mini-Ad vector replication. If the

preparation of mini-Ad vectors is not sufficiently pure, biochemical or other physical methods are utilized to achieve further purification.

BRIEF DESCRIPTION OF THE DRAWINGS

5 **Figure 1. The genome and transcription units of Ad5.** The Ad5 genome is approximately 36 kb, and is divided into 100 map units (mu). The dotted arrows represent early (E) transcription and the solid arrows represent late (L) transcription. The directions of transcription are indicated by arrows. Gaps between arrows indicate intervening sequences. The box represents location of the major later promoter and tripartite leader sequences (MLP). The solid triangle at 1 mu 10 represents the location of the packaging signal.

Figure 2. Development of the 293 cell. The 293 cell represents a human embryonic kidney cell transfromed with a fragment of Ad5 genomic DNA.

Figure 3. Methods for generating recombinant Ad vectors. A. The stow method, which is an *in vitro* recombination approach, uses ligation of the modified E1 region of the most left 15 end of the Ad genome to the isolated Xba I large fragment of the Ad genome (3.7-100 mu) of *dl*309, and transfection of the artificial adenoviral genome into 293 cells for production of new viruses. B. The plasmid recombination method, which generates Ad vectors through co-transfection of the shuttle and recombinant plasmids into 293 cells.

Figure 4. The principle of the mini-Ad vector system. Shown are the three major components 20 of the present invention: the helper Ad, the mini-Ad vector, and the Ad helper cell. E1-transactivation is provided by the helper cell and the helper Ad replicates itself and produces the late proteins required for formation of capsids. However, packaging of the helper Ad genome into the capsid is inefficient because of the attenuated packaging signal associated with the helper Ad genome. In the presence of the mini-Ad vector genome, the helper Ad 25 supports the DNA replication of the mini-Ad vector genome, which is preferentially packaged due to a wild-type packaging signal that has high affinity for the limited amount of packaging proteins. Further purification of the mini-Ad vector may be achieved using biochemical or physical methods, including but not limited to ultracentrifugation.

Figure 5. Comparison of the current Ad vectors with this invention. Depicted are the general 30 structures and complementary mechanisms of the current Ad vectors compared to those of the mini-Ad vector system.

Figure 6. The packaging signal of Ad5. Shown is the sequence and position of the packaging signal of Ad5 at the left end of the viral genome. Seven A-repeats have been identified as the packaging signal motifs. The consensus sequence of the A-repeat is proposed at the bottom of the figure.

5 **Figure 7. The prototype of the helper virus and the mini-Ad vector.** Shown is the general structure of the helper virus and the mini-Ad vectors.

Figure 8. Construction of the shuttle vector to generate the packaging attenuated helper AdHß. To construct GT5000, the mutant packaging signal sequence, mt Y, was amplified by PCR and substituted in a shuttle vector with an Ad5 sequence extended to 28.9 mu
10 (GT4004). A ß-gal expression cassette from pTk-ß was cloned into the E1-deletion of GT5000 to give the shuttle vector GT5001.

Figure 9. Generation of AdHß. The shuttle vector GT5001 (see fig. 8) was cotransfected with pJM17 in 293 cells. Recombination in the homologous 7 Kb region between these plasmids (9.24 to 28.9 mu of Ad5) yields a packageable virus with the left arm derived from GT5001.
15 The numbers in the left region of AdHß shown at the bottom correspond to Ad5 nt sequence and indicate extension of the double deletion in the packaging signal as well as in E1 where the ß-gal expression cassette is inserted. Two weeks after cotransfection, several blue plaques were isolated and the mutation in the packaging signal was analyzed by PCR with oligos 7 an 8. The size of the amplified fragment, 310 bp for the wild type packaging signal (wt Y)
20 and 177 bp for the mutant packaging signal (mt Y), can be distinguished in a 2% agarose gel as shown. 1 Kb ladder, 1 Kb DNA ladder marker from Gibco BRL (Gaithersburg, MD); wt Y and mt Y PCR controls where vDNA was extracted from an E1-deleted vector, Ad-CMVßgal, and from dl10/28 virus respectively; blue plaque, vDNA from a plaque that stained blue with X-gal; white plaque, vDNA from a plaque that did not stained blue with X-
25 gal (see fig. 10).

Figure 10. Identification of AdHß plaques versus other virus by staining with X-gal. [PCR amplification of the packaging signal of these plaques is shown in Figure 9.]

Figure 11. Amplification and characterization of AdHß. The packaging signal of AdHß was amplified after every passage due to the possibility that recombination with the endogenous
30 left Ad5 sequences present in 293 cells could generate a replication competent adenovirus (RCA, E1+) or an E1- adenovirus with wt . Wt was not detected in the passages previous

to the CsCl purification (4 to 8). At passage 9, when AdHß was purified, the viral DNA content was analyzed separately for every of the five bands of the gradient. In a 1% agarose gel (bottom left), almost no vDNA is observed in the upper three bands, indicating that they are formed mostly by empty capsids. Lower bands (4 and 5) are formed by full capsids. By
5 PCR the expected mutant packaging signal is detected in all the bands (bottom right). 1 Kb ladder marker (as in figure 9) at the left lane of every gel. Gel with vDNAs also contains l/Hind III marker.

Figure 12. Construction of mini-viral plasmids. These plasmids are constructed to determine the effect of various deletions of the adenoviral genome on packaging when complemented
10 with AdHß. All constructs contain the green fluorescence protein cDNA (GFP, striped box) driven by the CMV promoter with a ß-actin enhancer (thick arrow). M7.9 (bottom right) also has the neomycin cDNA and an internal ribosome entry site (IRES). The top six are derived from M32, which is a pJM17 derivative with a 10 Kb deletion in the middle of the Ad5 genome. The bottom two are derived from pBluescript-KS (Stratagene, CA) with the minimal
15 cis elements for replication and packaging of Ad5. Numbers correspond to Ad5 map units and indicate the deletion and insertion sites. 0/100 or 100/0 indicates the natural fusion of the inverted terminal repeats (ITR) of Ad5 DNA, Ad5 DNA, thick lane; plasmid backbone DNA, thin lane.

Figure 13. Schematic representation of the miniadenoviral vectors constructed for
20 **packaging.** At the top, the Ad5 transcription map and map units (mu) with the early (E) and late (L) transcription regions. MLP/TL: major late promoter and tripartite leader. The inverted terminal repeats (ITRs) and the packaging signal (Y) are the unique common sequences in all the miniAd vectors. The vectors are shown in a linear form as found after replication and in the capsid. The circular plasmids used for vector generation contain the
25 same sequences but fused head-to-tail by the ITRs. Every miniAd vector name refers to its size in Kb. M32 to M20 derive from pJM17 by progressive deletion of the central adenovirus genome. In these vectors the plasmid backbone (pBRX, not drawn) is located at 3.7 mu. M6.5 to vGnE5E3 are constructed in the pBluescript backbone (not drawn; located before the GFP-expression cassette) by insertion of neomycin cDNA and human genomic fragments
30 from chr. 4q11-22.

Figure 14. Two methods of complementation. To generate the mini-viral vectors two separate complementation protocols were used that gave similar yields. In the first method, the mini-Ad plasmid is cotransfected with viral DNA from AdHß, and the cells are cultured until a CPE is observed. In the second method, three days after an initial cotransfection of the mini-Ad plasmid with pBHG10, AdHß is added as virus, and cells are cultured until a CPE is observed.

Figure 15. Co-transfection of 293 cells with mini-viral plasmids. Nearly all cells were transfected by using a $CaPO_4$ modified protocol. Transfection with the M32 plasmid is shown 1 day after transfection. Right, bright field; left, fluorescence microscopy of the same field.

Figure 16. Minivirus-containing plaque. The presence of the minivirus (M32 here) is shown by the fluorescence of the plaque. Right, bright field; left, fluorescence microscopy of the same field.

Figure 17. Packaging efficiency of mini-viral vectors of different sizes. Packaging efficiency and amplification of miniAd vectors of different sizes. Thevirus produced after cotrasnfection is named as passage 0. The crude lysate was used to infect 293 cells to produce passage 1. 1 ml of crude lysate passage 1 was used to infect 106 293 cells and 24 h later the number of fluorescent cells were counted (transducing units/ml, dark columns). 24 h later CPE appeared and virus was extracted by freeze/thaw (crude lysate passage 2). Again, 1 ml of crude lysate passage 2 was used to infect 106 293 cells and 24 h later the number of fluorescent cells were counted (striped columns). The difference between passage 1 and 2 indicates an amplification yield of 5x and the difference between different miniAd vectors indicates the effect of the size in the packaging.

Figure 18. Purification scheme of M32. After amplification of M32 through several passages, the crude extract was CsCl-separated. The first gradient resulted in four bands: three upper and one lower. These were collected separately, dialyzed and used to infect 293 cells as shown in Figure 12. Fractions were collected from second separated gradients of the upper and the lower bands, and used to infect 293 cells as shown in figure 20.

Figure 19. Purification of M32 mini-Ad viruses (1): first CsCl gradient. M32 and AdHß co-purify in the band of higher density (number 4 or lower). The same wells used to check for fluorescence are later used to fix and stain with X-gal.

Figure 20. Purification of M32 mini-Ad viruses (2): fractionation of the lower band from the first CsCl gradient with a second CsCl gradient. An aliquot of 0.5 ml of every fraction was used to infect one well of a 96 well/plate with 293 cells at 60 % confluency. Initial fractions (1 to 6) did not contain M32 or AdHß (these fractions represent up to 3 ml of the gradient). 100 ml samples of fractions 7 to 16 reveal a large amount of M32 and AdHß (see panel B for ß-gal staining of the same fractions shown under fluorescence in panel A). Subsequent fractions (17 to 29) show a level of M32 similar to the previous fractions but the level of AdHß is approximately 10 times lower. Therefore, fractions 17-29 represent a 10-fold enrichment of M32 with respect to AdHß.

Figure 21. Ad5 packaging signal modification with GAL4 binding sites. The nucleotide sequences between the Xho I and Xba I sites are shown (design #1 and design #2). In design #1, there are two GAL4 binding sites before A repeat I and one GAL4 binding site between A repeat II and VI. In design #2, there two GAL4 binding sites before A repeat I and other two GAL4 binding sites after A repeat VII. The sequences underlined are 17 mer GAL4 binding sites. The sequences in italics are A repeats. The distance between center of each GAL4 binding site and A repeats is indicated.

Figure 22. Ad5 packaging signal modification with tetO sequence. The nucleotide sequences between the Xho I and Xba I sites are shown (design #1 and design #2). In design #1, there are two *tet*O sequences before A repeat I and one *tet*O sequence between A repeat II and VI. In design #2, two *tet*O sequences are present before A repeat I and further *tet*O sequences after A repeat VII. The sequences underlined are 19 mer *tet*O sequence. The sequences in italics are A repeats. The distance between the center of each *te*tO binding site and the A repeats is indicated.

Figure 23. Position and sequences of synthetic oligos for Ad Pac$^-$-GAL4 modification. Gal#1 to Gal#8 are synthetics oligos flanking the sequence between the Xho I and Xba I sites in design #1 and #2. The position and direction of each oligo is indicated by arrow bar. The sequences of Gal#1 to Gal#8 are listed.

Figure 24. Position and sequences of synthetic oligos for ad Pac$^-$-*tet*O modification. tet#1 to tet#10 are synthetics oligos to cover the sequence between the Xho I and Xba I sites in design #1 and #2. The position and direction of each oligo is indicated by arrow bar. The sequences of tet#1 to tet#10 are listed.

Figure 25. Construction of CMV-E1 mammalian expression vector. Adenovirus 5 sequences 462-3537 (AflIII-AflII Fragment) coding for E1A and E1B were blunt-end cloned into the EcoRV site of pcDNA3.

Figure 26. GFP expression and plaque formation in the E1-complementing cell line,
5 **A549E1-68.** After infection with an E1-deleted adenovirus, Ad5CA-GFP. The clear area in the center of this plaque is evidence of the CPE caused by E1-complemented virus amplification.

Figure 27. Southern blot analysis of G418^r A549E1 clones. Genomic DNA was digested with Hind III and probed with a 750bp E1 probe (PstI fragment). Lane 1: 1kb DNA ladder; Lane
10 2: A549; Lane 3: 293; Lane 4: A549E1-68; Lane 5: Subclone A549E1-68.3.

Figure 28. Morphology analysis of the new cell line. Morphological comparison of parental A549 cells (top panel) and the E1-complementing cell line, A549E1-68 (bottom panel).

Figure 29 Analysis of the E1 protein expression in transformed cell lines. A. Western blot analysis of E1A protein expression in A549 cells (Lane 1), 293 cells (Lane 2), and A549E1-
15 68 (Lane 3), using an E1A-specific monoclonal antibody (M73, Oncogene Science). B. Metabolic ^{35}S labeling and immunoprecipitation of E1B proteins in A549 cells (Lane 1), 293 cells (Lane 2), and A549E1-68 (Lane 3), using E1B p55-specific monoclonal antibody (Oncogene Science).

Figure 30. Design of the mini-adenoviral vector containing an integratable Factor VIII
20 **cassette.** Factor VIII cassette is contained between two AAV ITRs. A Rep expression cassette is positioned outside the integratable segment. In the target cells, the expression of Rep should provide targeted integration of the sequences flanked by AAV ITRs in the AAVS1 site in chromosome 19.

Figure 31. Design of plasmids GT9003, GT9004, GT9012 and GT9013. To construct plasmid
25 GT9003, Rep sequences from 193 to 2216 in the AAV genome were amplified by PCR (Pfu pol) from plasmid pSUB201, and cloned into pCRII (Invitrogen, CA). The resulting plasmid (GT9000) was digested with NotI and XhoI and a fragment containing an SV40 polyA site (Not-Sal I) was cloned in those sites. The resulting plasmid (GT9001) was digested with XbaI and blunt- ended with Klenow. A PvuII-PvuII fragment containing the whole AAV genome
30 was obtained from pSUB201 and subcloned in the blunted XbaI site in GT9001. This plasmid

(GT9002) was then cleaved with XbaI which removes the AAV coding sequences leaving the AAV ITRs. A neo-expression cassette (BamHI-BamHI) was then subcloned into GT9002 using XbaI and BamHI adaptors, giving rise to plasmid GT9003. Plasmid GT9004 was generated by removing the Rep coding sequences GT9003 using EcoRI. Plasmid GT9012 and GT9013 were generated by replacing the neo sequences (XbaI-XbaI) in GT9003 and GT9004, respectively, with a GFP expression cassette (SpeI-NheI).

Figure 32. Immunoprecipitation of Rep proteins in 293 and Chang liver cells. Cells grown in 10-cm Petri dishes were transfected with 10 mg of plasmids GT9001, GT9003, and GT9004 (see Figure 31 for details on construction of plasmids). Untransfected and GT9004-transfected cells were used as negative controls. Two days after transfection, cells were lysed and Rep proteins were immunoprecipitated using an anti-Rep monoclonal antibody (clone 226.7; ARP, Belmont, MA 02178) coupled to protein G-agarose, run on a 10% polyacrylamide gel and immunoblotted with the same antibody used in immunoprecipitation. Proteins were visualized by chemoluminiscence (ECL kit, Amersham). The migration of Rep78 and Rep52 proteins is indicated.

Figure 33. Southern blot of 293 clones transfected with plasmids GT9003 or GT9004. Fifteen mg of genomic DNA from several neo-resistant clones as well as a neo-resistant population (indicated as pool) were digested with EcoRI, electrophoresed, blotted onto a nylon membrane (Hybond-N, Amersham) and hybridized to an AAVS1 probe, spanning an 8Kb EcoRI-EcoRI fragment. The normal AAVS1 locus is indicated (panel A). Some GT9003 clones show a shifted band corresponding to disruption of one of the AAVS1 loci. Panel B shows the same membrane rehybridized to neo sequences.

Figure 34 . Southern blot of 293 clones transfected with plasmids GT9012 or GT9013. The conditions are as described in Figure 33. A) AAVS1 Southern blot. Some clones derived from plasmid GT9012 show rearrangements of AAVS1. B) Same blot rehybridized to GFP sequences.

Figure 35. Design of the episomal mini-adenoviral vector containing FVIII cassette. The mini-Ad vector is designed to form a circularized plasmid structure that contains episomal maintenance mechanism and the FVIII expression cassette, after the viral vector enters the target cells. The general structure of the vector has the following components: (a)

Recombinase expression cassette; (b) Origin of replication; (c) Human FVIII cDNA; (d) Recombinase target sites; (e) Adenovirus ITRs; and (f) Stuffer DNA sequence.

Figure 36. Scheme of pAlb12.5CAT plasmid. The plasmid contains the 12.5 kb human albumin promoter upstream of the chloramphenicol acetyl transferase gene (CAT) between
5 the EcoRI and HindIII site of the pBRCAT plasmid vector. The proximal promoter, and the enhancer $E_{1.7}$ and E_6 regions are shown.

Figure 37. Cloning of the 12.5 kb human albumin promoter into pBluescript vector. The EcoRI to AvaI 10.5 kb fragment and the 2.0 kb AvaI to HindIII albumin promoter fragments were isolated from pAlb12.5CAT and simultaneously ligated into a pBluescript vector
10 cleaved with EcoRI and HindIII to make GT4031.

Figure 38. Cloning of the hFVIII expression cassette into GT4031. The 7.5 kb human FVIII cassette was excised from plasmid GT2051 using XhoI and SalI and ligated into SalI-cleaved GT4031 to give GT2053, a human albumin promoter driven hFVIII expression vector.

Figure 39. Construction of an albumin promoter-FVIII minivirus plasmid. The adenovirus
15 5 ITR and packaging signal from GT2033 was excised with XhoI and cloned into the SalI site of GT2053. Plasmids having both orientations of the ITR were obtained. The insert in GT2061 is oriented with the unique SalI site proximal to the FVIII gene.

Figure 40. Restriction digest profiles of GT2053, GT2059 and GT2061. Digests show the expected banding patterns for BamHI, XbaI, ClaI and XhoI+SalI.

20 **Figure 41. Diagram of the albumin/alpha-fetoprotein gene region on chromasome 4.** Three regions which can serve as 3' recombination arms for homologous recombination are shown. They are Alb-E5 (a 3' region of the albumin gene), AFP-3 (a central region of the alpha-fetoprotein gene) and EBB14 (a region further 3' in the alpha-fetoprotein gene than Alb-E5).

Figure 42. Cloning scheme. Maps of three vectors containing different 3' homologous
25 recombination arms (Fig. 41) after cloning the arms into GT2061 (top) are illustrated.

Figure 43. Cloning scheme. Detailed cloning scheme for GT2063. The 3' 6.8 Kb XhoI fragment of the human albumin gene of clone pAlb-E5 was cloned into the SalI site of GT2061. A minivirus based on this vector is a potential *in vivo* therapeutic for FVIII gene therapy.

Figure 44. Restriction enzyme mapping. Agarose gel of the restriction enzyme digestion of the vectors leading up to GT2063, the final Albumin promoter-driven hFVIII with the 3' albumin homologous recombination arm as shown in Fig. 43. EcoRI and ClaI digests are shown for each of the indicated constructs.

5 **Figure 45. Scheme of generating the mini-AdFVIII virus.** Shown are two schema for generating the hFVIII minivirus. Scheme A, helper virus genomic DNA was cotransfected into 293 cells along with plasmid GT2063 on day 1. Transfection was by calcium phosphate precipitation . Six days after transfection, a CPE was observed and cell lysates were prepared. Subsequent infections were harvested on days 3 until passage 4 at which time the virus 10 preparations can be amplified 5-fold. Media is changed daily following infection. Scheme B, the plasmid pBHG10 was co-transfected with plasmid GT2063 using lipofectamine into 293 cells. Infection with helper Ad was performed 72 hours later at a multiplicity of 5 PFU. Four days later, a CPE was observed and cell lysates prepared. Infections were then performed at 1-fold inoculum volumes until passage 3 at which time the virus was amplified 5-fold. Media 15 was changed daily following infection.

Figure 46. PCR detection of the newly-generated hFVIII mini-Ad. Shown are the PCR results from passage 1 cell lysate from scheme A (lanes 3 and 4) and passage 1 supernatants on day 4 of infection from scheme B (lanes 5 and 6). The FVIII minivirus could be detected in both schemes (arrow). Primers were specific to hFVIII cDNA and amplifications were 20 performed on virus subjected to DNAse treatment prior to DNA extraction to remove any residual non-viral contaminating plasmid DNA. Lane 1 is 293 cell supernatant control. Lane 2 is 293 cell supernatant to which 30 ng of plasmid GT2063 was added and then DNAse treatment.

Figure 47. General structure of the first version of the anticancer super-Ad vectors. The 25 viral vectors consist of the Ad-helper and the super-Ad that contains multiple genes for cancer suppression and anticancer immunomodulation. The genes selected to be delivered are depicted in the diagram..

Figure 48. General structure of the second generation of the anticancer super-Ad vectors. The viral vectors consist of the Ad-helper and the super-Ad that contains multiple genes for 30 cancer suppression and anticancer immunomodulation. The general structure is similar to the first version of the vectors.

DETAILED DESCRIPTION OF THE INVENTION

Within this application, a *transcriptional regulatory region* is defined as any element involved in regulating transcription of a gene, including but not limited to promoters, enhancers and repressors.

A *DNA fragment* is defined as segment of a single- or double-stranded DNA derived from any source.

A *DNA construct* is defined a plasmid, virus, autonomously replicating sequence, phage or linear segment of a single- or double-stranded DNA or RNA derived from any source.

A *reporter construct* is defined as a subchromosomal and purified DNA molecule comprising a gene encoding an assayable product.

An *assayable product* includes any product encoded by a gene that is detectable using an assay. Furthermore, the detection and quantitation of said assayable product is anticipated to be directly proportional to the level of expression of said gene.

A gene expressed in a *tissue-specific manner* is that which demonstrates a greater amount of expression in one tissue as opposed to one or more second tissues in an organism.

An *effector gene* is defined as any gene that, upon expression of the polypeptide encoded by said gene, confers an effect on an organism, tissue or cell.

Heterologous DNA is defined as DNA introduced into an adenoviral construct that was isolated from a source other than an adenoviral genome.

A *transgene* is defined as a gene that has been inserted into the genome of an organism other than that normally present in the genome of the organism.

A *recombinant adenoviral vector* is defined as a adenovirus having at least one segment of heterologous DNA included in its genome.

Adenoviral particle is defined as an infectious adenovirus, including both wild type or recombinant. Said adenovirus includes but is not limited to a DNA molecule encapsidated by a protein coat encoded within an adenoviral genome.

A *recombinant adenoviral particle* is defined as an infectious adenovirus having at least one portion of its genome derived from at least one other source, including both adenoviral genetic material as well as genetic material other than adenoviral genetic material.

Stable gene expression is defined as gene expression that may be consistently detected in a host for at least a period of time greater than seven days.

A *treatable condition* is defined as a condition of an organism that may be altered by administration of a form of treatment including but not limited to those treatments commonly
5 defined as being of medicinal origin.

An *antigen* is defined as a molecule to which an antibody binds and may further inlcude any molecule capable of stimulating an immune response, including both activation and repression or suppression of an immune response.

A *tumor suppressor gene* is defined as a gene that, upon expression of its protein
10 product, serves to suppress the development of a tumor including but not limited to growth suppression or induction of cell death.

A *growth suppressor gene* is defined as a gene that, upon expression of its protein product, serves to suppress the growth of a cell.

An *oncogene* is defined as a cancer-causing gene.

15 An *immunomodulatory gene* is defined as any gene that, upon expression of its nucleic acid or protein product, serves to alter an immune reaction.

A *ribozyme* is defined as an RNA molecule that has the ability to degrade other nucleic acid molecules.

A *genetic condition* is defined in this application as a condition of an organism that is
20 a at least partially the result of expression of at least one specific gene including but not limited to the wild-type form of that gene and any mutant form of that gene.

1. The Basic Concept of The Mini-Ad Vector System

a. Composition of the system The mini-Ad vector system consists of three major parts:
25 (1) a packaging-attenuated helper Ad; (2) cognate Ad vectors having a minimal amount of the viral genome; and (3) Ad helper cell lines that provide functions of E1 *trans*-activation like 293 cells and/or regulation of packaging signal for the helper Ad. The helper Ad has all of the viral genes and elements that can be used for replicating itself and *trans*-complementing the mini-Ad vectors, except that it has an E1 deletion or substitution and a manipulated packaging signal that
30 can be used to control or discriminate the helper in the process of packaging the mini-Ad vectors. The mini-Ad vectors has only the ITRs and wild-type packaging signal as *cis*-elements for Ad

DNA replication and packaging. The rest of the mini-Ad vectors consists of transgene or heterologous DNA. The Ad helper cell lines similar to 293 cells have the Ad E1 genes and products that support helper Ad replication. The cells may also have control mechanisms for attenuating helper Ad packaging.

5 b. Mechanism of operation of the system This system is based on the fact that the packaging protein of Ad is a *trans*-acting factor present in low amount in the infected cells and is the rate-limiting factor in the packaging of Ad. As the wild-type packaging signal is recognized by the packaging protein with higher affinity than the engineered signal, packaging of the helper viral genomes with the mutations in the packaging signal can be partially or completely 10 suppressed in the presence of the mini-viral genomes with the wild-type packaging signal, ensuring preferential packaging of the mini-Ad vectors. In order to replicate and package the mini-Ad vectors at high-titer, the proteins for viral DNA replication and those for capsid assembly must be adequately provided from the helper virus. The designs of this invention allow the helper Ad to remain fully functional in replicating itself in helper cells utilized to produce 15 large quantities of Ad structural proteins. In the absence of the mini-Ad vector and without selection pressure of the packaging attenuation, the helper Ad is allowed to package itself slowly or ineffectively. In the presence of the mini-Ad vector, viral DNA replication proteins are also utilized to amplify the mini-Ad vector DNA to generate enough copies of the mini-Ad genome. The mini-Ad vector DNA, carrying the wild-type packaging signal, moves through the packaging 20 process to become incorporated into Ad virions, while the helper Ad DNA, competed off by poor recognition or low affinity of the packaging protein, remains completely or partially in the helper cells. Utilizing such a mechanism of packaging-attenuation for the helper Ad and packaging selection for the mini-Ad vectors, this system allows preferential propagation of the mini-Ad vectors. The mini-Ad vectors thus produced may not be 100% pure. The contaminating helper 25 Ad may be removed through other biological, biochemical, or physical methods including but not limited to ultracentrifugation through a CsCl gradient, if purification of the mini-Ad vectors is required for application (Fig. 4).

c. Capability of the system Two major features of the mini-Ad vector system of the present invention make it unique, sophisticated, and significantly advanced in contrast to any Ad 30 vectors currently available or reported (see Fig. 5 for comparison). First, the mini-Ad vectors have minimized immunogenicity and virtually no possibility of generating RCA. This is because

the mini-Ad vectors carry only a minimal amount of viral *cis*-element. The source of immunogenicity and cytotoxicity of the current Ad vectors has thus largely been removed. No Ad viral proteins will be produced in the target cells. Therefore, the cytotoxicity, induction of inflammatory response, and immunogenicity that are caused by the Ad viral proteins in the target
5 cells or presented partially on the surface of the target cells are eliminated. This system also has maximized the gene-delivery capacity for the Ad vector. Wild-type Ad has an average genome size of 36 kb. The maximal packaging capacity is about 105% of the genome, i.e. about 38 kb (27). Since the size of the viral *cis*-element in the mini-Ad vector can be trimmed to less than 1 kb, the capacity of the mini-Ad vectors to carry heterologous DNA can theoretically be up to 37
10 kb. The heterologous DNA may include but is not limited to transgene expression cassettes and regulatory elements. The expression cassettes may include but are not limited to single or multiple, bicistronic or polycistronic units. The regulatory elements may include but are not limited to DNA sequences for controlling transgene retention, integration, transcription, and vector targeting. All of these become possible due to the extra capacity for heterologous DNA
15 created in the mini-Ad vector.

2. The Packaging-Attenuated Helper Ad

a. The prototype structure of the helper The helper Ad vectors have a wild-type Ad genome containing an engineered packaging signal and an altered E1 gene (Fig. 7A). For safety reasons, the helper Ad must be defective in replication, such as the currently available E1-deleted
20 or substituted viral constructs. For the purpose of controlling packaging in the presence of the mini-Ad vector, the helper must be also defective in packaging (detailed below). Therefore, the general structure of the helper can be summarized as an Ad vector having a wild-type genome except that the E1 region and packaging signal are manipulated. However, the other essential regulatory genes of Ad such as E2 and E4 may also be manipulated. The viral genome may be
25 split into fragments in order to further disable the replication competence of the helper Ad or to reduce the genome size of the helper Ad for facilitating its separation from the mini-Ad vectors through CsCl-banding. As long as the titer of the helper Ad will not be significantly affected, both the defect in viral replication and attenuation in packaging of the helper Ad has been considered in the design of the structure of the helper Ad.
30 b. The general function of the helper Ad The primary function of the helper Ad is to supply capsids for packaging of the mini-Ad vectors. In order to fulfill this function, the helper

Ad must be able to replicate, although less efficiently than wild-type Ad. DNA replication and transcription of the helper genome should not be affected. Otherwise, the yield of the late gene products (the capsid proteins) will be affected accordingly and the titer of the mini-Ad vectors will be reduced. For certain applications, removal of the helper Ad from the mini-Ad may not be necessary. The stringency of packaging attenuation of the helper Ad can be greatly reduced. The helper Ad under this condition may also function to deliver transgenes together with the mini-Ad vectors. For example, in the mini-Ad vector system for anticancer treatment, the helper Ad can be designed to have functions not only to support the mini-Ad vector but also to carry genes (transgenes or Ad wild-type genes) for induction of anticancer immune responses.

c. The designs for packaging attenuation The purpose for attenuation of packaging the helper Ad is to reduce the potential for the helper Ad contamination in preparations of the mini-Ad vectors. This is especially important when a relatively pure batch of the mini-Ad vector is required for a particular application. The packaging function of the helper Ad is designed to be defective but not completely disabled, because the helper Ad must be able to replicate themselves, albeit slowly, in the absence of the mini-Ad vectors. The followings are the possibilities of designs for packaging attenuation of the helper Ad.

1. *Packaging signal mutation* The Ad5 packaging signal is composed of a repeated element that is functionally redundant (37) (Fig. 6). Partial deletions of the packaging signal elements have been shown to reduce the yield of mutant Ad from several fold to approximately a hundred fold as compared to that of Ad having a wild-type packaging signal (52). The design of the packaging signal mutation of the present invention may therefore incorporate a partial deletion of the motifs of the A-repeats from the wild-type Ad packaging signal.

2. *Synthetic packaging signal* Since the Ad5 packaging signal has a consensus A (adenosine) enriched motif (e.g. A-repeat: TAAATTTG), incorporation of an array of tandem repeats including but not limited to a selected A-repeat or any synthetic DNA motifs that may alter the affinity of the artificial packaging signal to the packaging proteins and alternate packaging of the helper Ad.

3. *Packaging signal interference* The Ad packaging signal is actually a specific DNA sequence that is recognized and bound by the packaging proteins. In order to interfere with the effective binding of the packaging proteins to the signal, other DNA binding sequences may be placed in proximity to within the A-repeat array of the packaging signal of the helper Ad. The

inserted binding sites allow high-affinity binding by their cognate DNA binding proteins that can positionally compete off the binding of the Ad packaging proteins to the Ad packaging signal, especially under the condition that the packaging signal of the helper Ad has been mutated and the affinity of binding by the packaging proteins has been reduced.

5 4. *Packaging signal relocation* The Ad packaging signal is naturally located at the left end of the wild-type Ad genome. There are reports that the packaging signal may be located at the right end and still be functional. This evidence indicates that the packaging signal is relocatable. A design that positions the engineered packaging signal at a non-wild-type location may be useful to further attenuate the packaging efficiency of the helper Ad. In addition, moving 10 the packaging signal to another region of the Ad genome may be helpful in minimizing any possibility of reversion of the helper Ad back to wild-type Ad through homologous recombination between the engineered packaging signal of the helper Ad and the wild-type packaging signal of the mini-Ad vectors.

5. *Further possibilities* To attenuate the packaging of the helper Ad in order to minimize 15 the contamination of the helper to a preparation of the mini-Ad vectors, two aspects can be considered: *cis*-elements and *trans*-acting factors. Therefore, other possible designs can be oriented towards these two aspects or any combination of these two aspects. The *cis*-elements are the A-repeats and the *trans*-acting factors are the packaging proteins. Further consideration should be a controllable mechanism of packaging without sacrificing the high titer output of the 20 mini-Ad vectors by the system.

3. The Mini-Ad Vector

a. The basic structure of the mini-Ad vector Ad vectors may be utilized as a circularized plasmids by fusion of ITRs (53). The simplest plasmid form of the mini-Ad vectors of the present invention is the circular DNA that contains an ITR fusion, plasmid DNA replication 25 origin, and polycloning sites. The ITR fusion includes the left end of the wild-type Ad from map unit 0 to 1 and the right end from map unit 99 to 100, in which the DNA replication origins of Ad are located in the each ITR and the wild-type packaging signal located adjacent to the left ITR.

b. The structural and functional possibilities of the mini-Ad vectors Based upon the basic 30 structure of the mini-Ad vectors, other DNA sequences and elements including but not limited to those listed below and in Figure 7B.

1. *Expression cassettes of transgenes* An expression cassette is a basic transcription unit. A simple expression cassette of a given gene is usually a linear DNA structure that consists of a promoter, the gene of interest, and a polyadenylation (polyA) signal. Within an expression cassette, two or more genes can be constructed as bi- or poly-cistronic units, as long as additional elements for translation or splicing of RNA are provided between the genes. Generally, mini-Ad vectors may include have one or multiple transgene expression cassettes.

2. *Functional elements for vector DNA retention* Elements that may assist in integration of the expression cassette into target cell genome or maintain the mini-Ad vectors as an episomal form in target cells.

3. *Regulatory elements for control of DNA transcription* Elements having transcriptional regulatory function including but not limited to enhancers, repressors, activator-binding sites, introns, and 5' or 3'-untranslated regions.

4. *Elements for vector and transgene targeting* Targeting can be achieved by several methods including but not limited to vector surface modification and tissue-specific expression. Tissue specific promoters may be utilized to drive gene expression in any specific cell type targeted *in vivo*.

5. *Further supporting elements* These may include but are not limited to DNA replication origins of prokaryotic or eukaryotic cells, plasmid or vector selection markers, and backbones of the vectors.

c. Designs for high titer production of the mini-Ad vectors High-titer production of the mini-Ad vectors is a major aspect of this invention. One advantage of Ad vectors over other viral vectors is that Ad particles are conducive to preparation of high-titer preparation stocks. High-titer propagation of Ad is mainly due to the large quantity of viral capsid protein production together with high copy numbers of the viral genome in host cells including but not limited to 293 cells. The followings are some of the factors that may be considered in designing methods for generating high-titer mini-Ad vectors.

1. *Enhanced DNA replication* Ad has its own enzymatic system for DNA replication. The E2 region proteins are the major *trans*-acting elements responsible for viral DNA replication. The replication origins are the cis-elements at the both ends of the viral genome. To enhance mini-Ad genome replication, sufficient quantity of E2 proteins expressed from the helper virus are provided. High-level expression of the E2 region proteins is ensured by the design of the

helper virus. Other such mechanisms for increase in copy numbers of the mini-Ad genome are also considered. Such mechanisms may include but are not limited to insertion of the the SV40 origin of DNA replication (53) into the mini-Ad genome to increase the copy numbers of the mini-Ad, as long as the SV40 T-Ag is expressed in the helper cell line.

5 2. *Enhanced packaging signal* A higher number or more efficient packaging sequences may be utilized by one of several methods including but not limted to incorporating a greater number of tandem repeats at one end or both, or generating synthetic packaging signals.

3. *Enhanced packaging process* The packaging process and mechanism of Ad are not yet fully understood by those skilled in the art. Whether DNA binding proteins other than the 10 packaging signal of Ad have synergistic roles for packaging is not certain. If so, then the sequences for DNA-binding proteins, referred to "anchorage points for packaging" and naturally existing within the Ad genome may need to be retained within the mini-Ad genome.

4. The Ad helper cell lines

a. The basic elements and general function of the Ad helper cells The cell lines utilized 15 in the present invention are preferably 293-like cells. A preferred embodiment of the host cell has the AdE1 fragment for trans-activation of the transcription program of the helper Ad genome incorporated into its genome. Unique from the E1 fragment in 293 cells currently available, the cell lines of present invention may include the E1 fragment is designed to comprise no overlapping sequences with the helper Ad genome. This allows for elimination of one of the 20 difficulties currently associated with Ad vectors: generation of wild-type Ad or replication-competent Ad (RCA) through homologous recombination. Other elements may include but are not limited to genes involved in support of high copy-number production of the mini-Ad vector, enhancing packaging of the mini-Ad vector, and attenuating the packaging of the helper Ad.

b. Assistance mechanisms for packaging attenuation of the helper Ad Methods with 25 which packaging attenuation of the helper Ad may be achieved may include but are not limited to expression of tetracycline-repressor (Tet-R), recombinase, as well as the specific design of the packaging protein. The Tet-R may bind to the engineered packaging signal of the helper virus comprising the proper binding site, the tet-operon (Tet-O) and repress packaging. Binding of Tet-R to Tet-O is controlled by tetracycline. Addition of tetracycline into the cell culture medium 30 results in binding of tetracycline to the Tet-R and prevents it from binding tet-O. Removal of the

tetracycline frees Tet-R for binding to the engineered packaging signal and serves to further attenuate packaging of the helper virus.

Expression of a recombinase such as Cre or Flp may inhibit packaging. The whole helper viral genome may be considered for further attenuation of the helper virus.

5 The specific design of the packaging protein may be altered by any of several methods including but not limited to utilization of a specific serotype or species difference in the packaging signal to differentiate packaging of the mini-Ad from the helper Ad provided the specific packaging protein of Ad is identified.

c. Assistance mechanisms for high-titer production of the mini-Ad vectors Modifications 10 of the mini-Ad vector designed to increase the copy number of the mini-Ad genome within a host cell are very important to the development of high-titer mini-Ad vectors. Expression of SV40 T-Ag (mutated T-Ag with no transforming activity) can be considered to make the copy number of the mini-Ad higher, if a SV40 DNA replication origin is incorporated in the backbone structure of the mini-Ad plasmid vector.

15 **5. Potential Applications of The Present Invention**

a. Delivery of genes for therapy of genetic diseases *in vivo* Large capacity is necessary for delivery of a large therapeutic gene or multiple genes as well as for transfer of regulatory elements and/or other related genes along with the primary therapeutic genes that will determine controllable or tissue-specific expression and may result in a more effective therapeutic effect. 20 An example includes but is not limited to cystic fibrosis in which controllable expression of several genes is required to optimize cystic fibrosis gene therapy. Gene therapy of Duchenne muscular dystrophy (DMD) is another example of a condition for which treatment would require a large capacity vector. For treatment of this disease, genes including but not limited to muscle and nerve growth factors may be required to be co-delivered in order to generate a complete 25 physiological effect to restore the muscle function of the patients.

b. Induction of host anti-cancer immunity through intratumoral injection of the vectors Ad vectors demonstrate high levels of infectivity in cultured tumor cells and different types of solid tumor models *in vivo*. This characteristic of the Ad vector has been utilized in the treatment of cancer. The efficacy of treatment depends upon the genes that are delivered by the vectors. 30 Multiple genes including but not limited to those having combined functions of tumor suppression and immunomodulation are utilized to optimize the anti-cancer effect. The mini-Ad

vector has the capacity to deliver multiple genes and is useful in constructing anti-cancer Ad vectors for intratumoral injection.

c. Modulation of host immunity by genetic modification of the graft cells or tissues Transplantation requires transient or permanent suppression of the host immunity. To deliver immune suppression genes into cells or tissues including but not limited to graft cells or graft tissues may be an alternative approach to the administration of immunosuppressive agents. Examples of genes encoding immune suppression proteins to be utilized in the present invention may include but are not limited to TGFb, IL-10, viral proteins HSV-ICP47 and CMV-US11, and secretable Fas-ligand proteins that may be delivered alone or in combination by the mini-Ad vectors of the present invention.

d. Modification of target cell function or regulation target cell growth *in vivo* by genetic modification Ad vectors have a distinct advantage over other viral vectors in that production of high titer stocks is possible, which is useful for *in vivo* gene therapy. Because the mini-Ad vectors contain only minimal amounts of cis-elements of the Ad genome, the immunogenicity of mini-Ad is minimized. Therefore, the mini-Ad vector will be useful for modifying target cell function or regulating target cell growth *in vivo* by genetic modification.

e. Specific delivery of transgenes to target cells or tissues *in vivo* by surface modification of the vectors The genes encoding the adenoviral hexon and fiber proteins are engineered to fuse with certain epitopes or ligands (e.g. the protein A that binds to Fc fragment of IgG) present on the target cell surface. These modified genes are incorporated into the recombinant viral genome for generation of the viruses having surface sites that interact with ligands that function as targeting agents on the target cell surface. The viral particles thus produced have tissue or cell recognition capabilities.

f. To be used for Ad-mediated vaccination via direct *in vivo* approaches For the purpose of vaccination, the immunogenicity of the E1-substituted Ad vectors may provide benefits, and has been used in development of Ad-based recombinant vaccines. Mini-Ad vectors utilized in this type of application use the helper virus including but not limited to E1-substituted Ad vectors as well as co-delivery of genes encoding antigens and immunogens that provide immunization.

g. To be used for ex vivo gene delivery Transient gene retention and expression associated with the use of conventional Ad vectors has prevented Ad from being widely used in ex vivo gene delivery protocols. The mini-Ad vectors, having DNA retention mechanisms, are

useful for this purpose. Also, the high infectivity of Ad in cultured cell lines make the mini-Ad vectors very effective gene delivery system for ex vivo approaches toward gene therapy.

h. To be used as tools for basic research and development of adenovirology and novel vector construction The mini-Ad vector system itself has a great value for basic adenovirology studies. The construction and demonstration of the feasibility and operation are already a breakthrough in the field. The helper Ad and the mini-Ad provide convenient tools for study of the Ad and its potential applications. This is particularly true for the mini-Ad vector. The characterization of the replication, packaging, and propagation efficiency of the mini-Ad will provide the field with important new information, which was previously unavailable.

i. To be used in combination with other methodology in the field of gene transfer and therapy Ad vectors have been used together with polylysine, liposome, and other conjugation materials as a gene delivery complex. The mini-Ad vectors can also be used with these compounds as well as any other copound that comprise the ability to serve as a gene delievery complex.

j. To be used for other purposes in the field of gene transfer and therapy The mini-Ad vector system has a great potential to be used for gene transfer and therapy in addition to what have been discussed above. The possibilities will come across along the further development of the field of gene transfer and therapy.

The following examples illustrate particular embodiments of the present invention and are not limiting of the specification and claims in any way.

EXAMPLES

Example 1: Construction and Characterization of The Packaging-Signal Mutated Helper Ad and Mini-Ad Vectors That Carry Green Fluorescence Protein (GFP) Reporter Gene

1.1 Generation of the Packaging-Signal Mutated Helper Ad

Several packaging signal deletion-mutants of Ad5 have been described (52). Mutant dl10/28 (also described as dl309-194/243:274/358) contains a deletion between nt 194 to 243 and between 274 to 358 of Ad5. dl10/28 virus was generated by the method of Stow (23) by ligation of a plasmid containing the left end of Ad5 with this double mutation (pE1A-10/28) and the rest of Ad5 genome (52). dl10/28 showed a 143-fold decrease in virus yield in a single virus

infection and, when co-infected with wild type virus, was not detected. We reasoned that with a helper virus containing the same mutation as dl10/28 we should be able to amplify the virus, although at low yields, and in the presence of mini-viral vector containing the wild type packaging signal the helper virus should remain unpacked.

5 The packaging signal was amplified by PCR from pE1A-10/28 using the following primers:

R7: 5'- GGAACACATGTAAGCGACGG

(nt 137 to 163 of Ad5 with AflIII site underlined) and

R8: 5'- CCATCGATAATAATAAAACGCCAACTTTGACCCG

10 (nt 449 to 421 with Cla I site attached).

The amplified 133 bp fragment was cut with AflIII and ClaI and used to substitute the corresponding sequence of the shuttle vector GT4004 (see Fig. 8 for this construction scheme). GT4004 derives from pXCX2 (5) by extending the Ad5 left region from XhoI site (nt 5792, 16 mu) until SnaBI site (nt 10307, 28 mu), therefore GT4004 contains the left end of Ad5 from 0
15 mu to 1.2 mu with the Afl III site at 0.38 mu, an E1 deletion from 1.2 mu to 9.2 mu with Cla I site in this deletion point and the rest of the left arm of Ad5 until 28 mu. This extended left arm increases the frequency of homologous recombination used to generate recombinant virus. GT4004 with the wild type packaging signal substituted by the deleted one was named as GT5000. The ß-gal expression cassette from pTkß (Clontech, Ca.) was cut as a SalI fragment,
20 blunted with Klenow enzyme and inserted into the blunted ClaI site of GT5000. The resulting plasmid, GT5001, contains therefore the double-deleted packaging signal and the E1 region of Ad5 replaced by the ß-gal gene driven by the Tk promoter (Fig. 8). This construct allows for detection of helper virus by X-gal staining.

To generate the helper virus the method described by Graham and Prevec was used (5)
25 (Fig. 9). An early passage of 293 cells obtained from ATCC, were grown in MEM-10% Horse Serum and seeded in 60 mm plates. At 30% confluence cells were cotransfected by $CaPO_4$ using 2 mg of GT5001 and 4 mg of pJM17 (5) per plate. Three days after cotransfection cells were overlaid with medium containing 0.5 % agarose and thereafter the medium above the overlay was changed every-other day. When plaques became visible, X-gal (40 mg/ml in DMSO) was directly
30 added to the medium to 100 mg/ml an let incubating overnight. Plaques producing the desired helper virus were identified by the blue color (Fig. 10). Blue plaques were fished and the agarose

plugs were resuspended in 1 ml MEM-10% FBS. 370 ml of this medium were processed for PCR amplification of the packaging signal as follows: 40 ml of 10 X DNase I buffer (400 mM Tris-HCl pH 7.5, 60 mM Cl_2Mg, 20 mM Cl_2Ca) (as a control of the treatment a tube with 0.5 mg of the shuttle vector was used) and 1 ml of DNase I (Boehringer M, 10 u /ml) were added and
5 incubated for 1 h at 37^0C. DNase I was inactivated and viral capsids were opened by adding: 32 ml EDTA (0.25 M), EGTA (0.25 M), 10 ml SDS (20%), 5 ml Proteinase K (16 mg/ml) and incubating at 56^0C for 2h. After one phenol:chlorophorm:isoamyl alcohol (1:1:1/24) extraction, 1 ml of yeast tRNA (10mg/ml) was added to help precipitation of viral DNA which was collected by centrifugation at 12000 rpm in a microcentrifuge and resuspended in 20 ml of H_2O. 5 ml were
10 used for a PCR reaction with primers R7 and R8.

One blue plaque with the desired deleted packaging signal was further amplified in 293 cells grown in DMEM-10% FBS. Hereafter named AdHelper-ßgal or AdHß (see Fig. 9). The virus was extracted at 48 h post-infection by centrifugation of the collected cells at 800 g for 5 min and three cycles of quick freeze and thaw of the cell pellet. This crude extract from X cells
15 was used to infect 3X cells (amplification scale was 1 to 3 in contrast to 1 to 20 for a virus with wild type packaging signal) (Figure 10). At every passage the deleted size of the packaging signal was verified by PCR of the supernatant (Fig.11). This deletion and the b-gal expression were stable in all the passages analyzed. At passage 9 AdHß was purified by CsCl. Purification was done by three cycles of freeze-thawing, layering the lysate onto a step gradient of 0.5 ml
20 CsCl 1.5 mg/ml + 2.5 ml CsCl 1.35 mg/ml + 2.5 ml CsCl 1.25 mg/ml, and centrifuging in a SW41 Beckman rotor at 10°C, 35000 rpm, 1h. The collected virus band was mixed with CsCl 1.35 mg/ml and centrifuged for 18 h as before. The virus band was dialyzed twice against PBS and once against PBS-10% glycerol, and stored at -80 ^{0}C. Five different bands were seen after the second gradient and every one was purified separately. Viral DNA was extracted from
25 purified virus by EDTA/SDS/Proteinase K treatment, phenol / chloroform extraction, and ethanol precipitation (same conditions as described above for PCR). In ethidium bromide gels, no DNA was detected in the three upper bands so we consider them to be mostly empty capsids. The two lower bands contained viral DNA and therefore were full capsids that, by restriction map analysis, were shown to correspond with AdHß. By PCR with R7+R8 oligos, the deleted
30 packaging signal was amplified from all bands, indicating that virus contained the desired attenuation. With this purified helper virus we test the packaging of different mini-viral vectors.

To determine the titer, which is expressed as a number of plaque forming units (PFU) per milliliter of virus in solution, the virus-containing solution was serially diluted in D-MEM 10% FBS (1:10 dilution until 10^{-12}) and used to infect 293 cells at 90% confluence (0.5 ml/well in 6 well-plates). After 1 h infection at 37 ^{0}C, the viral suspension was replaced by fresh medium. The
5 next day, cells were overlaid with medium containing 0.5% agarose, 0.025% yeast extract and 5 mM Hepes pH 7.4. Plaques were counted after 6 to 10 days. The titer obtained after amplification and purification of AdHß was about 10^9 PFU/ml (virus purified from 20 plates of 150 mm^2 and resuspended in a final volume of 1 ml). This titer is about 100x lower than that obtained with a similar viral vector containing the wt packaging signal.

10 **1.2 Construction of the plasmids for the mini-viral vectors.**

It has been shown that the linear adenovirus DNA, when covalently circularized head-to tail by its terminal ITRs can be grown as a plasmid in bacteria but it will replicate and produce virus when transferred into permissive human cells (53). Functional junctions have been naturally selected by transforming bacteria with circular DNA extracted from infected cells. Small
15 deletions in the joints were observed which presumably conferred stability to the plasmids by destroying the perfect palindrome that would result from the head-to-tail fusion of the ITRs of adenovirus DNA. The basic minivirus structure is therefore a plasmid that contains the left end of Ad5 (including the 103 nt-ITR and the packaging signal until nt 358) fused to the right end of Ad5 (at least including the 103 nt-ITR). The initial approach used to test the mini-viral vector
20 system included the generation of progressive deletions in plasmid pJM17 that contains a functional ITR fusion. pJM17 is a plasmid that contains the entire genome of Ad5 as a DNA molecule circularized at the ITR sequences and a pBR322 derivative, pBRX, inserted in E1A (providing the bacterial replication origin and ampicilin and tetracycline resistant genes) (55). When transfected in 293 cells, which complement the E1A defect, pJM17 replicates but is not
25 packaged because is too large (40.3 kb) to be packaged into the adenovirus capsid (maximum is 38 kb).

Examples of various mini-viral vectors demonstrated in the current literature as well as that of the present invention are illustrated in Figure 5. pJM17 was cut with AscI and religated obtaining pBRX-AscI. This removed from mu 43.5 to 70.2 of Ad5 which completely deletes E2A
30 (DNA binding protein) and L3 (hexon, hexon-associated proteins and 23K protease), and partially deletes L2 (penton base and core proteins) and L4 (hexon-associated protein, hexon-

trimer scaffold protein, and 33K protein). This deletion abrogates replication and capsid formation from the circular viral DNA, rendering it completely dependent on a helper virus that provides in trans a sufficient quantity of the required replication proteins. pBRX-AscI contains a unique Spe I site at 75.2 mu (L4) into which a 2.7 kb DNA fragment comprising a green fluorescence-protein (GFP) expression cassette was inserted to give M32 (Minivirus of 32 kB). This GFP-cassette is composed of a CMV enhancer/b-actin promoter (CA promoter), the *Aequorea victoria* GFP cDNA, and a SV40 polyA signal. The use of GFP in the mini-viral vector constructs was utilized in order to determine the presence of the vector in cells using the fluorescence microscopy. Flourescent microscopy represents one of several methods including but not limited to flow cytometry that may be utilized to detect cells expressing GFP. The presence of AdHß can be detected by the blue color of X-gal staining. To generate M31, M32 was cut with MluI and religated, this removes from 31.4 to 34.5 mu which partially deletes L1 (52K, 55K and penton-associated proteins). To generate M28, M32 was cut with MluI and AscI and religated, this removes from 31.4 to 43.5 mu which completely deletes L1 and the L2 portion that still remained in M32. To generate M26, M28 was cut with Rsr II and Spe I and religated, this removes from 30.9 to 75.2 mu extending the L1 and L4 deletions. To generate M23, M32 was digested with Nsi I and religated. The Nsi I fragment from 32.2 mu to the CA promoter (with a NsiI site next to the fusion with 75.2 mu), containing the GFP cassette, was religated so the Nsi I site of the CA promoter ligated to 5.5 mu and the Nsi I site at 32.2 ligated at 75.3 mu. This abrogates expression of all proteins between 5.5 to 75.3 mu including E2b (terminal protein, DNA polymerase) and IVa2 proteins. To generate M20, M23 was cut with Mlu I and Asc I, which removes the region from 34.5 to 43.5 mu of the Nsi I fragment of M23, and religated,.

Instead of trimming down a circularized full-length viral genome such as that in pJM17, other mini-viral vectors were constructed by subcloning the minimal cis elements necessary for replication and packaging, including the ITR sequences and the packaging signal, into a small plasmid such as pBluescript (Stratagene) and progressively adding the transgene cassettes and other elements that could improve the therapeutic potential of the viral vector such as elements for episomal maintenance or chromosomal integration (Figure 12, bottom). The head-to-tail fused ITRs and the packaging signal next to the left ITR (ITR/ITR+pac)were cut from pBRX-AscI with Eco47III (98.7 mu) and PvuII (1.26 mu) blunted and subcloned into SmaI - EcoR V of pBluescript, respectively. The resulting plasmid, pBS/MiniITR or GT4007, is a 3.8 minivirus

plasmid with no expression cassette and several unique restriction sites flanking the ITR/ITR+pac. Using Xho I and Kpn I, the GFP-expression cassette described above was subcloned into pBS/MiniITR to generate M6.5. An internal ribosome entry site (IRES) and a neomycin (neo) cDNA were then subcloned between the CA promoter and the GFP gene to
5 produce M7.9. A similar minivirus was generated comprising neo and GFP in two separate cassettes, M8.5: the Nru I-BstE II fragment from pREP9 (Invitrogen) containing the Tk promoter, neo cDNA and Tk pA, was blunted and subcloned into Stu I- EcoR I of M6.5. M8.5 was used to construct a larger miniAd plasmid in order to test the packaging of miniAd vector with a complete substitution of the adenoviral genome by exogenous DNA. As inserts we used genomic
10 fragments corresponding to the 3' half of the albumin gene and the 5' half of the alpha-fetoprotein gene. These fragments were chosen as potential arms with the prospect of studying homologous recombination in this site. We inserted these fragments upstream and downstream of the double GFP/neo expression cassette of M8.5. Therefore, in the resulting construct, pGnE5E3 (23.8Kb), the GFP and neo transgenes substitute the corresponding 10 Kb albumin- -
15 fetoprotein intergenic region present in the human genome. Figure 13 shows the structure of the minivirus to be obtained with the plasmids described above.

1.3 Generation and amplification of the mini-AdGFP vectors.

AdHß was utilized to support the replication and packaging of the various mini-Ad plasmids. It was important to determine whether the minivirus could be packaged. It was also
20 important to determine whether the size of the minivirus affected the packaging efficiency.

In adenovirus, 100% of the wild type length of DNA is most efficiently packaged, and as the genomic size increases to a maximum of 105% or decreases below 100%, packaging becomes less efficient. A lower limit of 69% (25 kb) has been suggested (32) when wild type adenovirus was used to complement the defective minivirus, but the use of an attenuated helper
25 virus allowed the amplification of a shorter minivirus.

To complement the mini-viruses of the present invention, two methods were utilized that each function with a similar efficacy (Fig. 14). In the first method, a CsCl-purified minivirus plasmid was cotransfected with the linear viral DNA extracted from purified AdHß. Note that the method utilized to purify the viral DNA is subjected to SDS and Proteinase K which destroys
30 the terminal protein responsible for priming replication. This method was utilized to avoid giving the helper virus a replicative advantage over the minivirus plasmid which also lacks the terminal

protein. Accordingly, complementation by direct infection with AdHß did not rescue minivirus. Cotransfection was accomplished using Ca_2PO_4 and 2 mg of mini-viral plasmid and 1 mg of viral DNA per well in a 6 well-plate with 293 cells at 50% confluence. After an overnight incubation in the transfection mixture, the medium was changed and the efficiency of transfection was assessed by examination of cells using fluorescence microscopy. With CsCl-purified plasmids this efficiency reached 100% irrespective of the size of the plasmids (Fig. 15). Six days post-cotransfection, CPE was observed and virus was harvested from the cells by three cycles of freeze and thaw. In the second method of complementation the minivirus plasmid was cotransfected with pBHG10, a circularized adenovirus plasmid similar to pJM17 incapable of being packaged due to a complete deletion of the packaging signal (56). This plasmid produces all the early proteins necessary for replication as well as the late proteins that form the capsid. When the minivirus is present in the same cell as pBHG10, it will also replicate and, as the minivirus contains the wild-type packaging signal, the miniviral vector will be the major nucleic acid encapsidated. However, when the minivirus is released to the neighbor cells it will not be amplified because is defective. Therefore, to amplify the minivirus, three days after the cotransfection, the cell monolayer was infected with AdHß at a multiplicity of infection (moi) of 10 plaque forming units (pfu)/cell. Three days after co-transfection, CPE was observed and virus was harvested by three cycles of freeze and thaw.

Regardless of the method of complementation, the lysate (passage 0 of the produced minivirus) was used to infect a fresh monolayer of 90% confluent 293 cells (using 1 to 3 amplification scale). The day after infection, the presence of minivirus was observed by fluorescence and the presence of helper confirmed by X-gal staining. If any helper virus was present in the lysate, further incubation of the cells would lead to the amplification of the mini-virus + helper mixture with the appearance of CPE (the new lysate of this monolayer will be considered as passage 1 of the minivirus). If no helper was present in the lysate, the minivirus alone would not be packaged and only by the addition of new helper would the CPE appear. Therefore the presence of the helper was assessed by X-gal staining and, with much higher sensitivity, by the appearance of CPE.

Following separate transfections with each of the minivirus constructs (M32, M31, M28, M26, M23, and M20), the appearance of plaques with GFP was observed (Fig. 16). This indicated that the complementation was possible for each of the plasmids tested. Following

infection of fresh 293 cells monolayers with the crude extracts of each virus, CPE was observed after 2 days indicating the presence of helper virus. The results of further passage of the minivirus demonstrated that in every passage a 5-fold amplification was produced, and that the packaging efficiency was proportional to the minivirus size (Fig. 17). A drop in efficiency of 2 fold per every 3 kb decreased vector size was observed. For example, the efficiency of packaging of M20 would be 2.48% of the wild type (being (36-x kb)/3=n the efficiency is 0.5^n). However, with M6.5, M7.9 and M8.5 no fluorescent plaques were found, indicating very inefficient or absent packaging (Fig. 17). This could reflect a packaging lower limit somewhere between 8.5 Kb and 20 Kb. However, it seems more probable that packaging still might take place between these limits but, according to the linear decrease observed, the 11.5 Kb size difference would result in a 7.6 fold less packaging efficiency and amplification may not then be possible.

Complete substitution of the viral genome by exogenous DNA was possible and whether this would affect the packaging efficiency was tested. A completely substituted miniAd of 23.8 Kb containing only the ITRs and the packaging signal of Ad5 was constructed. As exogenous DNA two expression cassettes in tandem, one for GFP and another one for neomycin, were flanqued by two long arms of albumin an -fetoprotein genomic DNA (Fig. 13). Packaging was demonstrated by the increasing number of GFP-positive 293 cells when the virus obtained after an initial complementation with AdH v DNA was passed. This transducing units titer was similar to that obtained with M23 (Fig. 17), indicating that exogenous DNA did not have a detrimental effect in the packaging efficiency when compared to Ad5 DNA.

1.4 Purification of the mini-AdGFP virus (M32)

Following passage of the minivirus, the titer increased until all cells became fluorescent following infection. This occurred, for example, at passage 4 of M32. When passage 8 was reached by continuously passing M32 at 1 to 3 amplification scale, enough virus was obtained to infect 75 plates of 150 mm^2. When CPE was apparent, the virus was extracted by three freeze/thaw cycles and purified by a CsCl gradient as described in example 1. In the gradient four bands were observed, three upper (and therefore lighter) bands and one thicker band in the middle of the centrifuge tube (see scheme in Fig. 18). Every band was collected separately by aspiration from the top of the tube, and dialyzed. Infection of 293 cells with every band and fluorescence observation or X-gal staining demonstrated that the mini-virus and helper virus were both present on the higher density band (Fig. 19). Based on the number of green and blue cells,

the amount of minivirus and helper was determined to be within the same range. The different size of the viral DNA present in M32 (32 Kb) and in AdHß (37.1 Kb) should make M32 slightly less dense than AdHß. To increase the mini-Ad vector to helper ratio the higher density band was separated in a 1.35 g/ml continuous CsCl gradient and fractions were collected from the bottom of the tube. 0.5 ml of every fraction was used directly to infect 293 cells at sub-confluency to check for fluorescent and blue cells after 24 h. Following a peak containing large amounts of AdHß (Fig. 20, fractions 7 to 16), a lighter fraction followed that revealed a 10-fold enrichment for M32 with respect to AdHß (fractions 17 to 29). Therefore, fractionation through CsCl may be utilized to decrease the amount of helper virus present in the mini-Ad preparations.

In summary, the results indicate that the helper used with the partial deletion in the packaging signal taken from the dl18/28 virus is able to complement the large deletions in the mini-viral vector system but it is still packaged in the presence of minivirus. This helper can be used when a pure population of minivirus is not critical, for example in an antitumoral vector system where a minivirus containing several therapeutic genes (interleukins and tumor-suppresser genes) can be combined with this helper containing another therapeutic gene. When higher mimi-Ad to helper ratio is required, this helper needs to be further attenuated in its packaging.

Example 2: Design of Packaging-signal interfered helper Ad

Since the packaging of adenovirus requires packaging proteins to bind the packaging elements (A repeats) (57, 52), and this invention introduces several specific DNA binding sequences adjacent to Ad5 packaging signals (A repeats) to further physically interfere helper virus packaging function. Two DNA binding sequences have been chosen: A. GAL 4 binding sequence (58); B. tetracycline operator sequence (*tet*O) (59, 60). GAL4 is a sequence-specific DNA-binding protein that activates transcription in the yeast *Saccharomyces cerevisiae.* The first 147 amino acids of GAL4 binds to four sites in the galactose upstream activating region UAS_G or a near consensus of the naturally occurring sites, the "17-mer" 5'-CGGAGTACTGTCCTCCG-3'or

5'-CGGAGGACTGTCCTCCG-3' (58). *tet*O comes from the Tn*10* -specified tetracycline-resistance operon of E. coli, in which transcription of resistance-mediating genes is negatively

regulated by the tetracycline repressor (*tet* R) which binds a 19-bp inverted repeat sequence 5'-TCCCTATCAGTGATAGAGA-3' in *tet* O (59, 60).

Based on the packaging signal mutation construct GT5000 (example 1, section 1), a synthetic sequence has been utilized to replace the sequence between Xho I and Xba I (nt 194, 0.5 mu to nt 452, 1.25 mu) of GT5000. Four synthetic sequences (Figures 21 and 22) have been designed. All four synthetic sequence contain the Ad5 packaging element (A repeats) I, II, VI and VII. Three or four repeats of 17-mer GAL4 binding sequences (5'-CGGAGTACTGTCCTCCG-3') (58) or 19-mer *tet*O sequences (5'-TCCCTATCAGTGATAGAGA-3') (60, 61) were introduced around or between these A repeats (Figures 21 and 22). Since the region between A repeats can affect packaging efficiency (57, 52), the distance between each A repeat is maintained as nearly integral turns of the helix, i.e. 10, 21 or 31 bp (Figures 21 and 22). Figures 23 and 24 show the synthetic oligo sequences and positions.

Example 3: Construction and Characterization of The Ad-E1 Helper Cell Lines

The majority of adenoviral vectors used in gene therapy applications were designed to have deletions in the E1 region of the adenovirus 5 (Ad5) genome. The E1 region, not including region IX, consists of 9% of the left end of Ad5 (1.2 - 9.8 map units), and encodes two early region proteins, E1A and E1B. Expression of E1A/E1B is required for virus replication and for expression of all other Ad5 proteins such as E2-E4 and late proteins (6). Deletion of E1 creates a replication-incompetent virus that, in theory, is silent for expression of all Ad5 proteins and expresses only the transgene of interest. Deletion of E1A and E1B is also of interest for safety reasons, since these two proteins, in combination, have been implicated in oncogenic transformation of mammalian cells (61-63). All of the Class I adenovirus vectors used to date in human clinical trials, as well as, the novel packaging-deficient helper virus described in Example 1 are deleted for E1.

E1-deficient adenoviral vectors are propagated in an Ad5 helper cell line called 293 (22). 293 cells were derived by transforming human embryonic kidney cells with sheared fragments of Ad5 DNA. Genomic analysis revealed that 293 cells contain four to five copies per cell of the

left 12% of the viral genome (including the entire E1 region) and approximately one copy per cell of 9% of the right end, the E4 region (64). While 293 cells are very efficient at producing high titers of E1-deficient adenovirus, they have the disadvantage that, due to the presence of extraneous Ad5 sequences integrated into the 293 genome (other than the E1 region), 5 recombination can occur with sequences in the E1-deficient adenovirus vector causing the production of E1-containing, replication-competent adenovirus (RCA). Depending on how early a passage the aberrant recombination event occurs during the amplification and propagation of the E1-deficient adenovirus, and which passage is used for large-scale production of the adenovirus stock, production of RCA in 293 cells can present severe ramifications for the safety 10 of human gene therapy trials (65). In addition to production of RCA, recombination in 293 cells can also cause deletions and rearrangements that effect transgene expression, thereby decreasing the titer of functional adenovirus particles. Recently, cell lines have been developed using defined Ad5 DNA fragments, including the E1 region, however these cell lines retain significant sequence overlap with homologous sequences in the E1-deleted adenovirus vectors, which allows 15 for undesirable homologous recombination events and the possibility for generation of RCA (66, 67).

3.1 Preparation of the plasmids for the cell lines

To eliminate the possibility of recombination with the adenoviral vector, a novel Ad5 20 helper cell line has been developed which harbors only the E1A/E1B sequences required for complementation, and does not contain any homologous sequences that overlap with regions in the E1-deficient adenovirus. A 3.1 kb DNA fragment between Afl III (462 bp) and Afl II (3537 bp) sites, which contains sequences encoding for Ad5 E1A and E1B, was cloned in two pieces, sequentially, into the superlinker vector, pSL301 (Invitrogen), as follows: First, an 881 bp Afl 25 III to XbaI fragment (Ad5 bp 462-1343) was cloned from pBRXad5KpnIC1 (a subclone of pJM17) into pSL301 (Afl III/XbaI). Second, a contiguous 2194 bp XbaI to Afl II (Ad5 bp 1343-3537) was cloned from pBRXad5XhoIC1 into the same vector. The resultant 3075 bp E1 fragment (in pSL301) contains the TATA box and RNA cap site for E1A, E1A coding sequence, complete E1B promoter, and E1B coding sequence, including the stop codon for E1B p55 30 protein, but not including region IX. The 3075 bp Afl III - Afl II E1A/E1B fragment (Ad5 bp 462-3537) was isolated, blunted with Klenow enzyme, and blunt-end ligated into the EcoRV site

of the mammalian expression vector, pCDNA3 (Invitrogen), under control of the CMV promoter/enhancer. This process generated an Ad5E1 expression vector, CMV-E1(Fig. 25).

3.2 Generation and characterization of the new cell lines

The CMV-E1 expression vector (including the G418 resistance gene, neo) was transfected using Lipofectamine (Gibco/BRL) into A549 human lung carcinoma cells and $G418^R$ colonies were isolated. Single-cell clones were screened for functional E1A/E1B expression; An E1-deleted adenovirus containing a green florescence protein (GFP) expression cassette under CMV/b-actin (CA) promoter, Ad5CA-GFP, was used to infect the A549-E1 clones. Three days post-infection, clones were screened for production of E1-complemented Ad5CA-GFP adenovirus by visual examination for cytopathic effect (CPE). One clone, A549E1-68, displayed 100% CPE in 3 days (similar to that observed for 293 cells). This clone also showed high infectivity, in that virtually 100% of the cells fluoresced green 24 hrs. post-infection (Fig. 26).

The high infection rate as well as rapid generation of CPE induced in this cell line is strong evidence that functional E1A/E1B proteins are being produced which are capable of promoting the replication and amplification of the E1-deleted Ad5CA-GFP virus. Southern Blot analysis using an E1 sequence-specific probe demonstrated the presence of the CMV-E1 transgene in A549E1-68, a subclone of A549E1-68 (E1-68.3), and 293 cells, but not in the parental A549 cell line (Fig 27). The morphology of the E1-transfected cells was significantly different from the parental A549 cell line. A549 cells, at sub-confluent density, grow as distinct single cells with an elongated, fibroblast-like morphology, whereas, the E1 cell line, A549E1-68, grows as colonies of cells with a more cuboidal morphology (Fig. 28).

A549E1-68 was compared with 293 cells for production of E1-deleted adenovirus (Ad5CA-GFP) by plaque assay and found to produce an equivalent titer of complemented virus (7×10^9 PFU for A549E1-68 vs. 9×10^9 PFU for 293). Immunoprecipitation and Western blot analysis using an E1A specific antibody (M73, Oncogene Science), revealed two E1A-specific bands with apparent molecular weights of 46kd and 42kd, corresponding to products expected from E1A 13S and 12S mRNAs (6), and identical in size to those observed in 293 cells (Fig. 29A). A549E1-68 produced a band of approximately 55 kd using a monoclonal Ab specific for E1B p55. This 55 kd, E1B-specific band, as well as secondary background bands, were observed in 293 cells also (Fig. 29B). Extra "background" bands found in both experimental and control lanes have been observed by other authors and have been attributed to co-immunoprecipitation

of a variety of proteins including, cyclins, p53, and Rb. Unlike A549E1-68 and 293 cells, the parental A549 cell line showed no expression of 46 kd, 42 kd, or 55 kd E1A/E1B proteins. It is clear that A549E1-68 not only expresses E1A and E1B, but that they are functional, since this cell line can complement for production of high titer, E1-deleted, recombinant adenovirus. To
5 prove that this new Ad5 helper cell line can complement without production of RCA, we are serially passaging E1-deleted adenovirus on A549E1-68 cells and testing the virus amplified during passaging, on parental A549 cells for production of E1-containing, replication-competent adenovirus (RCA) by CPE, as well as by using PCR primers specific for E1A/E1B sequences. This cell line will be used during propagation and scale-up of all E1-deleted adenovirus vectors,
10 to ensure that production lots are free of RCA.

Example 4: Construction of an integratable AAV-ITR/Rep system-based vector

Adeno-associated virus is a human non-pathogenic single-stranded linear parvovirus that
15 replicates only in the presence of a helper virus like adenovirus or herpes virus. However, in the absence of helper, AAV can integrate specifically in the host genome and be maintained as a latent provirus (68). The particular locus where AAV integrates has been located to chromosome 19q13.3-qter and named AAVS1 (69-72).

The mechanism of AAV integration has not been fully elucidated. However, two viral
20 elements have been implicated in this process: the AAV ITRs and two forms of the Rep viral proteins (Rep78 and Rep68). The AAV ITRs (Inverted Terminal Repeats) are palindromic sequences present in both ends of the AAV genome, that fold into hairpin structures and function as origins of replication. Several activities have been described for Rep78/68 proteins including sequence-specific DNA binding (73,74), sequence and strand-specific endonuclease activity (75),
25 and ATP-dependent helicase activity (75-77). These proteins can bind to a specific sequence in the ITR DNA and promote the process named *terminal resolution* by which the ITR hairpin is nicked and replicated (For a review, see Ref. 68). A Rep-binding motif and a terminal resolution site (*trs*) have been identified in both the AAV ITR and AAVS1 and demonstrated to promote *in vitro* DNA replication in the presence of Rep (78). It has also been shown that Rep68 protein
30 can mediate complex formation between the AAV ITR DNA and AAVS1 site *in vitro* (79). These findings suggest a model in which the DNA binding and endonuclease activity of Rep

along with limited DNA synthesis at the ITRs and AAVS1 sites would allow targeted integration of the sequences contained between the ITRs.

AAV has been considered as a candidate vector for gene therapy. However, the limited size of exogenous DNA that it can accept (4.2 Kb), the difficulty in getting high titers in large-scale preparations, and the loss of specific integration of the recombinant AAV have posed problems for the use of this virus as a gene therapy vector.

4.1 Construction of plasmids to test AAV/ITR-Rep integration system

Towards the incorporation of the AAV integration machinery in a mini-Ad vector (Fig. 30), we have developed and tested a plasmid vector that contains the Adeno-associated viral elements necessary for integration. In our design (Fig. 31), the vector consists of a Rep expression cassette (containing the viral endogenous promoter), as well as a cassette for expression of a reporter gene flanked by two AAV ITRs. The Rep expression cassette was obtained after PCR amplification of sequences 193 to 2216 in the AAV genome from plasmid pSUB201 (80). This fragment starts right after the ITR and extends through the p5 promoter and the Rep78 coding sequence (81).

4.2 Test of AAV/ITR-Rep integration system in culture cells

Expression of Rep from this plasmid in transiently-transfected 293 cells and an E1 non-expressing cell line (Chang liver cells) was tested by immunoprecipitation plus Western blot with specific antibodies. The results (Fig. 32) show that two different forms of Rep are produced in 293 and Chang liver cells. Rep78 appears as a doublet, which has been observed by other authors (82), while Rep52, product of p19 promoter, appears as a single band. Two other bands of sizes of approximately 68 kDa (Rep68) (faint) and 40 kDa (Rep40) are also be detected in 293 cells. Since the major splicing signal responsible for Rep68 and Rep40 production was not originally included in our design, it is possible that a minor splicing signal is functional in this construct (83). In Chang liver cells, two major forms are detected, Rep78 also as a doublet and Rep52, although the signal is stronger in 293 cells.

In order to test for the specific integration capability of these plasmids, a control plasmid was constructed by removing the Rep expression cassette (Fig. 31), but keeping the reporter gene expression cassette placed between two AAV ITRs. 293 cells were transfected with plasmids GT9003 or GT9004 and then selected for 12 days with G418 (0.5 mg/ml). G418-resistant colonies were isolated, expanded, and genomic DNA was extracted from different colonies by

the salt precipitation method (84). Genomic DNA was digested with EcoRI and analyzed by Southern blot with a probe for AAVS1. EcoRI was chosen because the AAVS1 locus is contained within an 8Kb EcoRI-EcoRI fragment. Figure 33 (panel A) shows that 50% of the resistant colonies analyzed which derived from plasmid GT9003 (Rep-expressing plasmid)
5 revealed rearrangements of at least one AAVS1 locus, as indicated by the presence of a shifted band in addition to the 8kb band corresponding to the normal sequence. Rearrangements were not observed in the colonies derived from plasmid GT9004, indicating that this phenomenon is dependent on the expression of Rep. These results suggested that Rep was able to drive specific integration of the transgene. The membrane was then rehybridized to a specific probe for neo
10 (Figure 33, panel B). The pattern of bands obtained indicated that some AAVS1 rearrangements correspond to neo (ex. clone 2L2) but also suggested that random integration events occurred frequently in the clones analyzed, possibly favored by the selective pressure applied.

4.3 Test of AAV/ITR-Rep integration system without selective pressure

In order to rule out this possibility, we performed another set of experiments with
15 plasmids GT9012 and GT9013 (Fig. 31). In these plasmids the reporter gene is GFP (*Aequorea victoria* green fluorescent protein). This reporter makes cells suitable for isolation using methods including but not limited to sorting and single-cell cloning by flow cytometry, thereby eliminating effects of selective pressure imparted by the neo expression cassette. 293 cells were transfected with either plasmid. One day after transfection, cells falling into a given range of
20 fluorescence (thus eliminating variability due to differences in transfectability) were sorted by flow cytometry and single-cell cloned in 96-well plates. Two to three weeks after sorting, colonies were scored for fluorescence. Three independent experiments were performed and the results are shown in Table 2-1. The cloning efficiency (number of colonies developed per total number of seeded wells) showed some variability for GT9013-derived cells, but was generally
25 constant for those transfected with plasmid GT9012. Approximately 50% of the colonies derived from plasmid GT9012 were fluorescent and maintained their fluorescence in subsequent passages, whereas 8% of those derived from plasmid GT9013 showed any fluorescence. The fluorescence intensity was dim, an observation consistent with the integration of one or few copies of the GFP expression cassette into the host cell genome. Interestingly, some colonies
30 showed a mosaicism in GFP expression. One explanation for this could be that the integration

event occurred after the sorted cell started division giving rise to two different populations. In a parallel experiment cells were transfected with plasmid pCA-GFP that contains a GFP expression cassette alone (no viral sequences). Fluorescent colonies were not detected after sorting plus single-cell cloning (Table 2-1). Taken together, these results indicate that the efficiency of integration is enhanced by the presence of AAV ITRs but is 4-5 fold higher when Rep is expressed. To further analyze targeted integration of GFP in AAVS1, several colonies (fluorescent and non-fluorescent) were grown and genomic DNA was extracted as described above. Figure 34 (panel A) and Table 2-2 summarize results obtained by Southern blot with a probe for AAVS1. Rearrangements of AAVS1 are indeed detected in several colonies derived from plasmid GT9012 whereas no rearrangement is observed in GT9013-derived colonies, thus indicating that the presence of Rep is necessary for targeted integration. This membrane was then probed for GFP to check the correspondence with the rearranged bands (Figure 34, panel B). Parental cell line 293 was negative, as expected. Five clones showed bands over 8 Kb matching those obtained with AAVS1, therefore indicating specific integration of GFP in AAVS1. Surprisingly, a band of 7 Kb was detected in several clones. This band is not present in the parental cell line DNA, thus ruling out the possibility of a cross-hybridization with human-related sequences. A band of similar size was also present in the GT9003 and GT9004 derived clones (Figure 33). Although an definite explanation for this is not available, some possibilities inlcude but are not limited to the following: 1) presence of plasmid DNA in episomal form (EcoRI digestions should render a 7 Kb band regardless of the plasmid used), although it is unlikely since the cells were kept in culture for approximately 4 weeks before DNA was extracted. Digestions with another enzyme and hybridization with a probe for the plasmid backbone should provide the necessary information to answer this question; 2) Not only the sequences in between the ITRs are integrated but the whole plasmid does. However, if only one ITR is used for integration, this would not explain a 7 Kb band unless there is another EcoRI site adjacent to the site of integration, or another binding site for Rep in the plasmid is used (i.e. p5 promoter). A third possibility is that there is another preferred site of integration different to AAVS1.

Altogether, the results shown above indicate that plasmids containing AAV ITRs and Rep can integrate at high frequency in the host genomic DNA and suggest that this design is useful for the integration of sequences delivered by adenoviral vectors (Fig. 30).

Table 2-1. Results of single-cell cloning experiments.

		GT9012	GT9013	pCAGFP
Experiment 1	Cloning efficiency*	92/192 (48%)	116/192 (60%)	
	Integration efficiency**	43/92 (47%)	12/116 (10%)	
Experiment 2	Cloning efficiency	42/96 (44%)	26/96 (27%)	
	Integration efficiency	23/42 (55%)	2/26 (8%)	
Experiment 3	Cloning efficiency	93/192 (48%)	25/192 (13%)	51/96 (53%)
	Integration efficiency	37/93 (40%)	1/25 (4%)	0/51 (0%)

5

* indicates number of colonies per number of wells seeded.

** indicates number of colonies showing fluorescent cells two weeks after sorting per number of colonies.

10

Table 2-2. Summary of AAVS1 rearrangements in the 293 clones analyzed by Southern blot (data obtained from Southern blot, Figure 34).

Fluorescent GT9012 clones	Non-fluorescent GT9012 clones	Fluorescent GT9013 clones	Non-fluorescent GT9012 clones
7/11	1/3	0/3	n.d.

15

Example 5: Designs of The Episomal Mini-Ad Vectors

As an alternative approach to provide elements that will allow long-term expression of
20 the transgene delivered by the mini-viral vector, we have designed a site-specific recombinase-

based system that permits excision of an auto-replicative episome from the mini-viral sequences upon infection of target cells.

Site-specific recombinases have been extensively used to manipulate DNA. Site-specific recombinases catalyze precise recombination between two appropriate target sequences, cleaving
5 DNA at a specific site and ligating it to the cleaved DNA of a second site (for a review, see Ref. 85). Several systems have been identified and characterized such as the cre/loxP system from bacteriophage P1 (86) or FLP/FRT from yeast (87). The recognition sites (loxP and FRT) for both recombinases (cre and FLP) share a common structure: they have two inverted repeat elements (recombinase binding site) flanking a central core region (site of crossing-over). The
10 orientation of the target sites (as defined by the core region) is responsible for the final outcome: recombination between two parallel sites on the same molecule results in excision of intervening sequences generating two molecules, each one with a target site. Recombination between two antiparallel sites results in inversion of the intervening sequence. Recombination between two parallel sites in diferent molecules results in the integration of sequences flanked by target sites.
15 Since excision is an intramolecular event, it is favored over integration.

In the design of the present invention, recombinases will be used to excise sequences having an eukaryotic origin of replication (ori). Mammalian ori sequences and binding factors have not been characterized to date. However, some viral ori sequences and viral proteins required for initiation of replication have been characterized and incorporated in plasmid vectors,
20 some examples of which including but not limited to SV40 ori/T-Ag from simian virus 40 (88) and oriP/EBNA-1 from Epstein-Barr virus (89). These elements have allowed the generation of plasmids that replicate autonomously in eukaryotic cells and are stably maintained upon selective pressure. Plasmids containing oriP and expressing EBNA-1 protein replicate once per cell cycle (90) and are lost when selective pressure is removed from cells in culture. However, there is no
25 *in vivo* data about the stability of episomal plasmids in nondividing cells, such as hepatocytes. One should expect that in nondividing cells (i.e. differentiated cells) and without selection, an episome could remain stable for a long period of time. It is believed by the inventors of the present invention that the incorporation of ori sequences in the mini-viral DNA will permit a extended expression of the transgene in nondividing cells.

30 The episomal minivirus elements include but are not limited to (Fig. 35):

a) Recombinase expression cassette: recombinase must be expressed only in target cells, because inappropriate expression in the cells used to generate the virus will promote the excision of the sequences contained between two recombination sites. For this reason, expression is tightly controlled by either adding binding sequences for transcriptional repressors upstream of the
5 promoter (for instance, tetO) or through the use of tissue-specific promoters (ex: albumin promoter, factor VIII promoter).

b) Origin of replication (ori): must include the sequence to initiate or begin replication of DNA and any other element required for replication (ex: DNA binding protein recognizing origin sequences).

10 c) Transgene: may be any therapeutic or reporter gene flanked by a recombination site (5') and a polyA signal sequence (3'). It will be expressed only in target cells upon circularization of the DNA.

d) Recombinase target sites: two sites are necessary in parallel orientation, one being placed between the promoter and the recombinase cDNA and the other upstream of the
15 therapeutic gene cDNA.

e) Adenovirus ITRs: necessary for replication and packaging of the minivirus.

f) Stuffer DNA sequence: if necessary to increase the size of the minivirus up to a packageable length. The stuffer DNA sequence may be any DNA fragment of any length.

Under this design, the recombinase is not expressed while amplifying the minivirus.
20 When the mini-viral vector is delivered to target cells, the promoter is functional, recombinase is expressed and the sequences contained between two recombinase target sites are excised and circularized. The recombinase promoter turns into the transgene promoter and the presence of the origin of replication allows stable maintenance of the plasmid, therefore assuring stable expression of the transgene.

Example 6: Construction of hFVIII Mini-Ad Vectors

Hemophilia A results from deficiencies in the expression or function of the blood coagulation factor VIII in affected individuals and can be treated by infusions of the normal clotting factor either obtained from plasma concentrates or purified from cultured cells engineered to express FVIII through recombinant DNA techniques (91). Therapeutic benefit can be achieved at 5-10% of the normal plasma levels which average between 200 and 300 ng (1 Unit) per milliliter (92). Greater than 30% of the normal plasma levels would allow for near normal lifestyle (93). Gene therapy approaches for the future treatment of hemophilia must overcome several difficulties but has much potential (94).

The human FVIII gene cDNA is about 9 kb in length with a coding region of 7.2 kb (95, 96). The size of the gene limits its use in most viral vectors such as retrovirus, adenovirus and adeno-associated virus vectors although the B domain can be deleted to reduce the size of the gene for insertion into retrovirus or adenovirus vectors. However gene expression and propagation of retroviral particles capable of gene delivery has been hampered by regions within the gene which result in silencing or instability of the mRNA transcripts (97, 98). Successful expression has been reported using retroviral vectors and B-domain deleted human FVIII (99, 100). Recently an E1 substituted adenovirus vector incorporating the B-domain deleted FVIII cDNA under control of the murine albumin promoter has been used to achieve levels of human FVIII expression in mice at up to 1.5 times the normal plasma levels (101). Expression was sustained for 7-9 weeks with a gradual decline which correlated to loss of the adenovirus vector DNA in the liver tissue. Such decline is attributable to immune responses directed to adenovirus proteins expressed from the vector after gene delivery in addition to human FVIII (102).

Adenoviruses may represent the ideal vectors for delivery of FVIII due to the fact that (1) I.V. injection of adenovirus results in targeted gene expression to the liver; (2) Expression of FVIII from the liver has achieved significant plasma levels of FVIII; (3) The liver is a major site of synthesis of FVIII in normal individuals; and (4) Liver transplants have successfully corrected hemophilia in the previously hemophiliac recipients. Furthermore, significant advances could potentially be achieved if the full length FVIII was delivered as opposed to the B-domain deleted forms and the vectors could include additional elements which would allow for stabilization of transgene expression in the target cell either as an episome (artificial chromosome) or by facilitating integration of the introduced gene into the cell genome.

The novel adenovirus vector system described of the present invention retains such advantages. The vectors of the present invention have the insert capacity needed to deliver the full length FVIII cDNA coding region as well as devices such as homologous recombination arms or viral integration mechanisms designed to promote stability of the vector in target cells. Additionally, adenovirus proteins are not expressed from the minivirus vectors that carry the transgene, thereby reducing detrimental immune responses.

6.1 Expression cassettes of the FVIII cDNA

The size capacity of the Ad-mini vector allows for the use of large promoter and cDNA coding regions that exceed the size capacity of conventional Adenovirus vectors. Since FVIII delivery is targeted to the liver, it is reasonable to express FVIII using a highly active liver specific promoter including but not limited to the human albumin gene promoter. A human albumin promoter region of 12.5 kb in length was obtained from the University of Calgary (Dr. Tamaoki's laboratory). Three regions within the 12.5 kb promoter segment were determined to have a significant influence on the overall promoter strength (103 Hayashi et al, 1992). These are the proximal region having the TATA box (550 bp), an enhancer region at -1.7 kb and a second enhancer region at -6.0 kb (Fig. 36). Combined, these regions approximate the total promoter effect of the entire 12.5 kb human albumin promoter. For expression of human FVIII, a plasmid was constructed using the Bluescript vector as the plasmid backbone. The EcoRI/AvaI 10.5 Kb fragment from pAlb12.5CAT (Fig. 36) was co-ligated with the AvaI/HindIII proximal promoter fragment into EcoRI/HindIII cleaved Bluescript vector to give GT4031 (Fig. 37). The 7.2 kb full-length human FVIII cDNA with the 5' flanking SV40 immediate early intron and a 3' flanking SV-40 poly-adenylation signal was obtained as an XhoI/SalI fragment derived from an expression plasmid GT2051 and was cloned into the SalI site of GT4031 to generate plasmid GT2053 (Fig. 38). The XhoI fragment derived from plasmid GT2033 containing the minimal ITR region and Ad packaging signal was cloned into the SalI site of GT2053 to generate the albumin/hFVIII minivirus plasmids GT2059 and GT2061 (Fig. 39). The restriction map profile of the minivirus plasmids containing the two different orientations of the ITR/packaging signal fragment are shown in Figure 40.

6.2 Homologous recombination arms of the expression cassette

Homologous recombination is one mechanism that can be employed for functional gene insertion into target cells for stable gene expression. Incorporation of the human FVIII cDNA into

the genomic DNA of target cells can be tested using homologous recombination. To do this, large segments or arms of cellular DNA derived from the human albumin gene or human alpha-fetal protein were used (103 Hayashi et al., 1992, 104 Urano et al., 1984). The albumin promoter of 12.5 kb functions as the upstream homologous recombination arm while a number of downstream fragments of greater than 6 kb were prepared as potential 3' recombination arms. A figure of the albumin gene, an intergenic region and the alpha fetal protein (AFP) gene regions is shown (Fig. 41). The general structure of the expression cassette in plasmid GT2061 with the 12.5 kb Albumin promoter at the 5' end and several regions serving as the 3' homologous recombination arms is summarized in Figure 42. These vectors will serve as homologous recombination replacement vectors since the orientation of the arms are in the same direction as the sequences in the normal human genome. A construct with the 3' XhoI recombination arm derived from the human albumin gene, pAlb-E5 cloned into the unique SalI site of GT2061 is shown in Figure 43. Restriction enzyme digestion of the vectors leading up to the final Albumin promoter-driven hFVIII with the 3' albumin homologous recombination arm is shown in Figure 44. The plasmid GT2063 was constructed by cloning the plasmid pE5 XhoI albumin gene fragment into the unique Sal I site of GT2061.

6.3 Generation of the FVIII mini-Ad vectors

The mini-Ad vectors were constructed as described above. Briefly, FVIII mini-Ad vectors were prepared by first cloning the 12.5 kb EcoRI to HindIII human albumin promoter region into the Bluescript vector. The resulting promoter clone contained a unique SalI site into which a human FVIII cDNA flanked by XhoI and SalI and containing the SV40 early intron at the 5' end and the SV40 polyadenylation signal at the 3' end was cloned. The resulting plasmid contained unique Sal1 and Xho1 sites at the 3' end of the human FVIII gene close to the 3' end of the polyadenylation site. The adenovirus minimal ITR and packaging sequence contained within an XhoI fragment derived from clone GT2033 was then cloned into the SalI site to generate GT2061. The albumin gene 6.8 kb arm from pAlb-E5 was cloned into the unique Sal I site of 2061 to generate GT2063. This clone is now being tested as an adenovirus mini-virus vector by transfecting into 293 cells for packaging and propagation.

A adenovirus clone pBHG10 which lacks the Ad packaging signal and the E1 region but does code for the remainder of the Adenovirus proteins was co-transfected into 293 cells along with the minivirus clone. Rescue of the Ad-minivirus genome is performed by infection of the

cultures with E1 substituted helper virus with attenuated packaging function. Since both the Ad-helper and minivirus genomes can be packaged, both viral forms are carried by the particles generated using this technique but the ratios of each may vary experimentally.

6.4 Test of the mini-AdFVIII in cell lines

5 Clones pBHG10 and the mini-hFVIII vector GT2063 were transfected into 293 cells by lipofectamine transfection (Gibco/BRL). After 48 hours, infection with an attenuated helper virus was performed as described in section 1.3. Cytopathic effect (CPE) was observed after 6 days and passage 0 virus stocks were prepared by freeze thaw of the cell pellets. At passage 1 (P1), cells were infected with the primary passage 0 stocks (1:1). Supernatants collected at 24 10 hours post infection were positive by PCR specific to the hFVIII cDNA. The scheme for the generation of the FVIII mini-Ad is depicted Figure 45 and the PCR results and methods are described in Figure 46. After 6 days CPE was again observed and freeze-thaw (P2) lysates prepared. The Mini-Ad virus is being tested by PCR for packaged GT2063 minivirus and for functional hFVIII. The levels of expression of hFVIII in 293 cells is expected to be low since the 15 human albumin promoter does not function well in these cells as determined by CAT assays and assays of human functional FVIII after transfection with GT2061 by the calcium phosphate precipitation.

In another approach (Fig. 45), the helper-virus genome purified from virus was co-transfected into 293 cells using the calcium phosphate precipitation method. After CPE was 20 observed cell free freeze-thaw lysates were prepared and used for infection of fresh cells. After 24 hr hFVIII specific to the GT2063 virus was detected in the cell supernatants. The data are consistent with propagation of a helper/ GT2063 minivirus mixture.

6.5 Test of the mini-AdFVIII in animal models

Upon propagation and large scale growth of the Ad-minivirus, tests are conducted in 25 C57Black and Balb-C mouse strains. Different titers of each mini-Ad vector (from 108 to 5 x 109 pfu/mouse) in PBS are injected via the tail vein. Vector infectivity, level of hFVIII expression, and persistence will be analyzed at different time points on the liver and blood samples from the animal models.

30 **Example 7: Design of The Mini-Ad for Treatment of Cancer**

Currently, one of the most effective approaches to the treatment of cancer using gene therapy is to alter the tumor-host relationship and facilitate the recognition and destruction of malignant cells using the immune system. In the tumor bearing individual, the lack of an effective immune response may be due in part to either weak tumor cell immunogenicity, lack of immune co-stimulation, or a tumor-specific immunosuppressive environment. Cytokine-mediated gene transfer of tumor cells offers one strategy to augment the immune system to mount a more effective antitumor response (105). In recent years a number of cytokine genes have been isolated, cloned and characterized. Systemic administration of certain of these immunomodulators, such as IL-2, has resulted in a proportion of antitumor responses. However, toxicities have accompanied the use of many of these biologics owing to the high concentrations needed to generate clinical effects. The combination of significant undesired effects and marginal therapeutic outcomes from systemic administration has stimulated efforts to genetically engineer tumor cells to produce the cytokines themselves (106).

In animal models, gene modified tumor cells have been used as vaccines to stimulate antitumor responses (105, 107). The appeal of tumor directed cytokine gene transfer is that the cytokine, produced locally, is immunologically more efficient and does not cause systemic toxicity. Tumor antigens expressed on neoplastic cells presented with high local concentration of the cytokine(s), would create an immunological microenvironment impossible to reproduce with exogenous cytokine administration. This immunological microenvironment created by the cytokine producing tumor cells has been efficient in generating cytotoxic T lymphocytes. In a number of different animal models, cytokine producing tumor cells have been shown to be effective in decreasing the tumorgenicity and increasing the expression of immunologically important molecules (105, 107). The initial antitumor rejection appears to be accompanied by a nonspecific inflammatory response. However, rejection of cytokine secreting tumor cells has in most instances led to the generation of systemic, tumor specific immunity that is T cell dependent.

A requirement for preexisting tumor immunogenicity has not been established for most gene transfer models; however many well-characterized tumor cell lines are highly differentiated and immunogenic. In some systems, nonimmunogenic tumors have been shown to generate immunity after cytokine gene transfer. Furthermore, most tumor directed gene transfer models do not lend themselves to investigations in which the host is treated in the presence of an existing

tumor burden because the rapid growth of these malignancies provides little time for immunotherapeutic intervention (107).

Recent research has demonstrated that the reduction of TGFb secretion by tumor cells may be a significant approach to cancer gene therapy (108, 109). In one set of experiments Fakhrai et al., used antisense to TGFb to inhibit the expression of that cytokine in a rat gliosarcoma cell line. Immunization of tumor-bearing rats with the antisense modified tumor cells resulted in significant survival of animals compared to animal's immunization with tumor cells modified with control vectors. Using a different approach Isaka et al., was able reduce the amount fibrotic disease in rats, by transfecting skeletal muscle with a cDNA encoding decorin. Decorin is a small proteoglycan that inhibits the expression of TGF . Thus, two different approaches to inhibit TGFb expression has shown efficacy in two different models of cancer or pre-cancer.

In addition, new evidence demonstrates that co-stimulation of T cells by B7 has both a positive and negative effect on T cell activation (110). Other co-stimulatory molecules for T cells such as ICAM-I, LFA-3 and VCAM-I have also been implicated in the induction of appropriate anti-tumor responses (111). A general consensus among those skilled in the art is that the most important of these co-stimulatory signals is provided by the interaction of CD28 on T cells with its primary ligands B7-1 (CD80) and B7-2 (CD86) on the surface of antigen presenting cells (112). In a variety of model systems tumor cells transfected with the B7 cDNA induced potent antitumor responses against both modified and unmodified tumor cells. CTLA-4, a molecule also expressed on T cells, binds B7-1 and B7-2 with much higher affinities than CD28. Results of several studies demonstrate that CTLA-4 acts as a negative regulator of T cell responsiveness, and raises the possibility that blocking the inhibition delivered by the CTLA-4-B7 interaction might augment the T cell response to tumor cells and enhance antitumor activity. Leach et al., demonstrated that injecting antibody to CTLA-4 resulted in the rejection of tumors including pre-established tumors in a mouse model (110). This demonstrates the care must be used in designing gene transfer experiments such that the desired effects are not masked by other potential deleterious effects.

The genetic basis of cancer includes abnormalities in oncogenes and/or tumor suppressor genes. Both types have been the targets of cancer gene therapy. Because the cancer-related defects of tumor suppressor genes are usually mutations or deletions, the strategy in tumor

suppressor gene therapy thus far developed has been gene replacement therapy, in which a wild-type tumor suppressor gene is transferred into cancer cells to restore the normal function of the defective gene or induce tumoracidal effect (113). The human tumor suppressor genes that have been cloned and characterized include *Rb*, Wilms tumor (*WT1*), and neurofibromatosis (*NF1*), which are involved in pediatric cancers; adenomatosis polyposis coli (*APC*) and deleted in colon cancer (*DCC*), which contribute to colorectal cancer; and *p53,* which is found in mutated forms in a wide range of human cancers (for a review, see Ref. 114). Recently, two major events occurred in the area of identification of new tumor suppressor genes or cancer susceptibility genes. First, two highly related members of the cyclin-dependent kinase (cdk) inhibitor family, termed *p16* (major tumor suppressor 1, *MTS1*) and *p15* (*MTS2*), were isolated from the chromosomal region 9p21 (115, 116). Second, a strong candidate for the breast and ovarian cancer susceptibility gene *BRCA 1* was identified (117). While *p16* was shown to be deleted or mutated in a wide range of cancer cell lines, *p15* was shown to be a potential effector of TGF-b-induced cell cycle arrest (118). Among all of those tumor suppressor genes, the p53 gene is the one that has thus far been utilized for gene therapy of cancer (119).

Our current effort on gene therapy of cancer is to combine tumor suppresser gene and immunomodulation gene therapy of cancer with the introduction of other molecules such as, tumor antigens, MHC molecules, cell adhesion molecules and other immunomodulating factors. The followings are the general description of the two designs of the anticancer super-Ad vectors.

7.1 Construction the first version of the anti-cancer super-Ad vectors

Several combinations of immune molecules and genes may be utilized in the constrcution of anti-cancer super Ad vectors. The mini-Ad vectors may carry of the multiple genes that function to suppress tumor growth or induce host anticancer immune responses. This type of vectors is called anticancer super-Ad vectors. The first version of the super-Ad vector will carry four double expression cassettes for human p53 cDNA, GFP marker gene, human IL2 cDNA, human GM-CSF cDNA, human B7-1 cDNA, human IL7 cDNA and human IL12 p35 and p40 cDNA. It also contains minimum sequence of left and right Ad5 ITR and Ad5 packaging sequence (total 660 bp) and about 18 kb genomic sequence of human a-fetoprotein gene to reach over 30 kb size (Fig. 47). Cassette 1 includes a CMV promoter, a Human p53 cDNA, an EMC-IRES, a GFP gene and a SV40 pA. Cassette 2 includes an EF promoter, a human GM-CSF cDNA, an EMC-IRES, a human IL 12 cDNA and a bovine growth hormone pA. Cassette 3

includes an SV40 promoter, human B7-1 cDNA, an EMC-IRES, human IL7 cDNA and SV40 pA. Cassette 4 includes a tk promoter, a Human IL12 p35 cDNA, an EMC-IRES, a human IL12 p40 cDNA and a bovine growth hormone pA (Fig. 47).

7.2 Construction the second version of the anti-cancer super-Ad vectors

5 A second version of the anti-cancer super Ad vectors has a similar structure to that of the first version, including adenovirus inverted terminal repeats at both the 5' and 3' ends and four discrete expression cassettes. Several combinations of regulatory molecules and genes may be utilized in the constrcution of anti-cancer super Ad vectors. The examples described below are not in any way limiting to the types of mini-Ad vectors that may be constructed to regulate the 10 growth of a tumor cell. Each expression cassette is flanked at the 5' end by a unique promoter. In addition, each expression cassette incorporates two genes linked by the encephalomyocarditis virus internal ribosome entry site sequence for cap independent translation of the "distal" gene. The genes shown for this vector include cytokine genes as represented by IL-2, IL-7, and GM-CSF; a tumor suppresser gene as represented by p53; immune cell co-stimulatory molecules as 15 represented by B7-1 and ICAM-1; and molecules that can reverse the immune suppression often associated with cancers, anti-TGFb and SCA to CTLA-4. To increase the size of the vector so that the vector will be efficiently packaged into progeny virus, we have included a "stuffer DNA" of human alpha-fetoprotein. The stuffer DNA may include any DNA fragment of any length. The general structure of the second version of the anticancer super-Ad vectors are shown in 20 Figure 48.

Example 8: Other Designs for Improvement of the system

1. Mini-Ad vectors having targeting capability. Multiple mechanisms may be utilized to target gene expression to a specific cell type or tissue. One such mechanism involves 25 transcriptional targeting of a cell type, cell type subset or a specific tissue. Transcriptional targeting includes the use of a transcriptional regulatory unit that drives gene expression in only a certain type of cell or tissue. Such a transcriptional regulatory unit is referred to as being tissue-specific. A mini-ad vector is designed to incorporate a tissue-specific transcriptional regulatory unit driving expression of a reporter or effector gene. In this manner, expression of the reporter 30 or effector gene under control of the tissue-specific transcriptional regulatory unit will be detected at a higher level in those specific tissues in which the transcriptional regulatory unit is

active. It may be preferable to restrict gene expression to a certain cell type or tissue. Therapeutic genes are often toxic if expressed in high amounts. Regulation of gene expression to specific tissues, then, may serve to protect the host from the adverse effects of high level gene expression of certain therapeutic genes.

5 A further method to direct tissue-specific gene expression would be to utilize a helper virus that encodes a cell surface protein reactive to a ligand on a cell type of interest. For instance, a helper virus may be engineered to express a ligand for a cell surface receptor. Upon packaging of the recombinant packaging-competent DNA construct of this invention, an recombinant adenoviral particle that binds to a receptor on the surface of a cell is produced. A
10 further example would include a recombinant adenovirus that expresses an antibody or a fragment of an antibody on the surface of its viral coat. Such a recombinant virus may be produced by engineering a packaging-deficient helper virus to express an antibody or antibody fragment as a fusion or a separate protein on its viral coat. Upon infection of a cell transfected with a DNA molecule encoding an at least an adenoviral packaging sequence and at least one
15 reporter or effector gene, recombinant adenoviral particles having an antibody or antibody fragment reactive to a cell surface molecule on a target cell are produced. In this manner, recombinant adenoviral particles will specifically bind to those cells in the host that express cell surface molecules reactive to said antibodies or antibody fragments.

2. The mini-Ad vectors that have local immune suppression function. Certain
20 autoimmune disorders result from the inappropriate immune reactions. One method that may be utilized to prevent, halt or slow the autoimmune reaction is to direct expression of immunomodulatory proteins at the site of such reactions. This may be accomplished by application of adenoviral particles constructed from a mini-Ad genome as demonstrated within this application. Genes encoding certain cytokines or chemokines may be expressed and such
25 expression may result in an attenuation of the immune reponse. This attenuation in the immune response would then lead to an alleviation of the symptoms of the autoimmune reaction. A further example may include the attenuation of an allergic reaction. An antigen known to cause an allergic reaction may be encoded by a mini-Ad vector. Upon expression either low levels or extremely high levels of the antigen, driven by the mini-Ad vector delivered to a cell by a
30 recombinant adenoviral particle, tolerance may result. Also, expression of the antigen may be directed to tissues in which expression of the antigen may induce tolerance. Such a tissue may

include the developing thymus. Following desensitization, the host into which the recombinant adenoviral particle was delivered will not exhibit an allergic reaction upon interaction with that antigen. In this manner, a form of immunosuppression has been achieved by administration of the recombinant adenoviral particle carrying engineered mini-Ad DNA molecule.

5 **3. The mini-Ad vector that hybridize with other elements.** It will also be possible to utilize the mini-Ad vectors disclosed in this application to prevent or eliminate viral infection and replication within a host. Mini-Ad vectors can be designed such that viral certain genetic processes may be interfered with or eliminated. The mini-Ad vectors may be designed to express antisense nucleic acids that interfere with viral replication at the transcriptional or translational 10 stage of infection. Interference may be promoted by the expression of antisense RNA or DNA including that which binds to messenger RNA or binds to DNA after integration of a viral genome to prevent transcription. Also, ribozymes may be designed that target certain viral transcripts for destruction. "Decoy" molecules may also be encoded by a mini-Ad vector. Such decoys may function by binding to transcription factors required for viral trasncription such that 15 the trasncription factors are no longer available for binding to and driving trasncription of genes required for viral gene expression and replication.

4. The mini-Ad vector that can be used for vaccination. Mini-Ad vectors may be engineered to drive expression of certain antigens or immunogens that will serve to generate immunity in the organism in which expression takes place. Mini-Ad vectors may be designed that 20 drive expression of bacterial or viral genes that induce an immune reaction resulting in immunity. For example, a coat protein from a retrovirus such as HIV may be encoded by a mini-Ad vector. Upon infection of cells in a host with a recombinant adenoviral particle comprising said mini-Ad vector, immunity to the HIV virus may ensue. Mini-Ad vectors may also be designed to drive expression of cancer-specific antigens. Upon infection of cells in a host with a recombinant 25 adenoviral particle comprising a mini-Ad vector directing expression of a cancer antigen, immunity to that type of cancer will follow. Optimally, such immunity will result in widespread eradication of the primary tumor as well as other metastases and micrometastases that exist throughout the treated organism. Additionally, mini-Ad vectors may be designed that encode antigenic molecules derived from a parasite. Following administration of a recombinant 30 adenoviral vector comprising the mini-Ad vector, immunity to infection by said parasite will result.

While a preferred form of the invention has been shown in the drawings and described, since variations in the preferred form will be apparent to those skilled in the art, the invention should not be construed as limited to the specific form shown and described, but instead is as set forth in the claims.

1. An isolated nucleic acid molecule for generating immunity comprising an adenoviral (Ad) inverted terminal repeat (ITR) fusion sequence, a packaging signal, a transcriptional control region operably linked to a DNA sequence encoding at least one immunogen, all operatively associated for generating an infectious, replication-defective recombinant adenoviral vector wherein the remaining portion of said nucleic acid molecule does not encode an adenoviral protein.

2. An isolated nucleic acid molecule of claim 38 wherein said adenoviral ITR fusion sequence comprises the Adenovirus type 5 (Ad5) right ITR and the Ad5 left ITR.

3. An isolated nucleic acid of claim 39 wherein said ITR fusion sequence comprises Ad5 map unit 98.7 to map unit 1.26.

4. An isolated nucleic acid of claim 39 wherein said ITR fusion sequence is the Eco47III (98.7 mu) to PvuII (1.26 mu) fragment of pBRX-AcsI.

5. An isolated nucleic acid molecule of claim 38 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

6. An isolated nucleic acid molecule of claim 39 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

7. An isolated nucleic acid molecule of claim 40 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

8. An isolated nucleic acid molecule of claim 41 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

9. An isolated nucleic acid molecule of claim 42 wherein said infectious agent is selected from the group consisting of a virus, bacteria, fungi, prion, mycoplasma and a parasite.

5 10. An isolated nucleic acid molecule of claim 46 wherien said virus is a human papilloma virus or a human immunodeficiency virus.

11. An isolated nucleic acid molecule of claim 45 wherein said immunogen is the human papilloma virus E6 protein or the human papilloma virus E7 protein.

10

12. An isolated nucleic acid molecule of claim 42 wherein said cancer antigen is selected from the group consisting of MAGE, MUC1, MUC-2, CEA, MART, SP100, HER-2/NEU, PSA, tyrosinase, Melan-A, RAS, Rb, WT1, NF1, APC, DCC, and p53.

15

13. An isolated nucleic acid molecule of claim 42 wherein said receptor is associated with autoimmune disease.

14. An isolated nucleic acid molecule of claim 42 wherein said ligand is Beta Human
20 Chorionic Gonadotropin.

15. An isolated nucleic acid molecule of claim 38 wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding at least one immunogen selected from the group consisting of an infectious agent antigen, a cancer antigen,
25 a receptor protein antigen, and a ligand antigen; and a nucleic acid sequence encoding at least one immunomodulatory protein.

16. An isolated nucleic acid molecule of claim 52 wherein said immunomodulatory protein is selected from the group consisting of an interleukin, an interferon, a
30 chemokine, IL-2, IL-3, GM-CSF, B7-1, B7-2, IL-7, IL-12, IL-12 p35, IL-12 p40, IL18, ICAM-1, LFA-3, and VCAM-1.

17. A replication-defective, recombinant adenoviral particle for generating immunity comprising a nucleic acid molecule comprising an adenoviral inverted terminal repeat (ITR) fusion sequence, a packaging signal, a transcriptional control region operably linked to a DNA sequence encoding at least one immunogen, all operatively associated for generating an infectious, replication-defective recombinant adenoviral vector wherein the remaining portion of said nucleic acid molecule does not encode an adenoviral protein.

18. A replication-defective, recombinant adenoviral particle of claim 54 wherein said adenoviral ITR fusion sequence comprises the Adenovirus type 5 (Ad5) right ITR and the Ad5 left inverted terminal repeat.

19. A replication-defective, recombinant adenoviral particle of claim 55 wherein said ITR fusion sequence comprises Ad5 map unit 98.7 to map unit 1.26.

20. A replication-defective, recombinant adenoviral particle of claim 55 wherein said ITR fusion sequence is the Eco47III (98.7 mu) to PvuII (1.26 mu) fragment of pBRX-AcsI.

21. A replication-defective, recombinant adenoviral particle of claim 54 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

22. A replication-defective, recombinant adenoviral particle of claim 55 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

23. A replication-defective, recombinant adenoviral particle of claim 56 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

24. A replication-defective, recombinant adenoviral particle of claim 57 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

5 25. A replication-defective, recombinant adenoviral particle of claim 58 wherein said infectious agent is selected from the group consisting of a virus, bacteria, fungi, prion, mycoplasma and a parasite.

26. An isolated nucleic acid molecule of claim 62 wherein said virus is a human
10 papilloma virus or a human immunodeficiency virus.

27. An isolated nucleic acid molecule of claim 61 wherein said immunogen is the human papilloma virus E6 protein or the human papilloma virus E7 protein.

15 28. A replication-defective, recombinant adenoviral particle of claim 61 wherein said cancer antigen is selected from the group consisting of MAGE, MUC1, MUC-2, CEA, MART, SP100, HER-2/NEU, PSA, tyrosinase, Melan-A, RAS, Rb, WT1, NF1, APC, DCC, and p53.

20 29. A replication-defective, recombinant adenoviral particle of claim 61 wherein said receptor is associated with autoimmune disease.

30. A replication-defective, recombinant adenoviral particle of claim 61 wherein said ligand is Beta Human Chorionic Gonadotropin.

25

31. A replication-defective, recombinant adenoviral particle of claim 54 wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding at least one immunogen selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein, and a ligand antigen; and a nucleic acid sequence
30 encoding at least one immunomodulatory protein.

32. A replication-defective, recombinant adenoviral particle of claim 68 wherein said immunomodulatory protein is selected from the group consisting of an interleukin,

an interferon, a chemokine, IL-2, IL-3, GM-CSF, B7-1, B7-2, IL-7, IL-12, IL-12 p35, IL-12 p40, IL18, ICAM-1, LFA-3, and VCAM-1.

33. An isolated nucleic acid molecule for generating immunity comprising an adenoviral inverted terminal repeat (ITR) fusion sequence, a packaging signal, a transcriptional control region operably linked to a DNA sequence encoding at least one immunomodulatory protein, all operatively associated for generating an infectious, replication-defective recombinant adenoviral vector wherein the remaining portion of said nucleic acid molecule does not encode an adenoviral protein.

34. An isolated nucleic acid molecule of claim 70 wherein said adenoviral ITR fusion sequence comprises the Adenovirus type 5 (Ad5) right ITR and the Ad5 left inverted terminal repeat.

35. An adenoviral particle of claim 71 wherein said ITR fusion sequence comprises Ad5 map unit 98.7 to map unit 1.26.

36. An adenoviral particle of claim 71 wherein said ITR fusion sequence is the Eco47III (98.7 mu) to PvuII (1.26 mu) fragment of pBRX-AcsI.

37. An isolated nucleic acid molecule of claim 70 wherein said immunomodulatory protein is immunostimulatory.

38. An isolated nucleic acid molecule of claim 71 wherein said immunomodulatory protein causes immunostimulation.

39. An isolated nucleic acid molecule of claim 72 wherein said immunomodulatory protein is immunostimulatory.

40. An isolated nucleic acid molecule of claim 72 wherein said immunomodulatory protein is immunostimulatory.

41. An isolated nucleic acid molecule of claim 74 wherein said immunomodulatory protein is selected from the group consisting of an interleukin, an interferon, a chemokine, IL-2, IL-3, GM-CSF, B7-1, B7-2, IL-7, IL-12, IL-12 p35, IL-12 p40, IL-18, ICAM-1, LFA-3, and VCAM-1.

42. An isolated nucleic acid molecule of claim 70 wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding an immunogen.

43. An isolated nucleic acid molecule of claim 79 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

44. An isolated nucleic acid molecule of claim 80 wherein said infectious agent is selected from the group consisting of a virus, bacteria, fungi, prion, mycoplasma and a parasite.

45. An isolated nucleic acid molecule of claim 81 wherien said virus is a human papilloma virus or a human immunodeficiency virus.

46. An isolated nucleic acid molecule of claim 80 wherein said immunogen is the human papilloma virus E6 protein or the human papilloma virus E7 protein.

47. An isolated nucleic acid molecule of claim 80 wherein said cancer antigen is selected from the group consisting of MAGE, MUC1, MUC-2, CEA, MART, SP100, HER-2/NEU, PSA, tyrosinase, Melan-A, RAS, Rb, WT1, NF1, APC, DCC, and p53.

48. An isolated nucleic acid molecule of claim 80 wherein said receptor is associated with autoimmune disease.

49. An isolated nucleic acid molecule of claim 80 wherein said ligand is Beta Human Chorionic Gonadotropin.

50. A replication-defective, recombinant adenoviral particle for generating immunity comprising an adenoviral inverted terminal repeat (ITR) fusion sequence, a packaging signal, a transcriptional control region operably linked to a DNA sequence encoding at least one immunomodulatory protein, all operatively associated for generating an infectious, replication-defective recombinant adenoviral vector wherein the remaining portion of said nucleic acid molecule does not encode an adenoviral protein.

51. A replication-defective, recombinant adenoviral particle of claim 87 wherein said adenoviral ITR fusion sequence comprises the Adenovirus type 5 (Ad5) right ITR and the Ad5 left inverted terminal repeat.

52. A replication-defective, recombinant adenoviral particle of claim 87 wherein said ITR fusion sequence comprises Ad5 map unit 98.7 to map unit 1.26.

53. A replication-defective, recombinant adenoviral particle of claim 87 wherein said ITR fusion sequence is the Eco47III (98.7 mu) to PvuII (1.26 mu) fragment of pBRX-AcsI.

54. A replication-defective, recombinant adenoviral particle of claim 87 wherein said immunomodulatory protein is immunostimulatory.

55. A replication-defective, recombinant adenoviral particle of claim 88 wherein said immunomodulatory protein is immunostimulatory.

56. A replication-defective, recombinant adenoviral particle of claim 89 wherein said immunomodulatory protein is immunostimulatory.

57. A replication-defective, recombinant adenoviral particle of claim 90 wherein said immunomodulatory protein is immunostimulatory.

58. A replication-defective, recombinant adenoviral particle of claim 91 wherein said immunomodulatory protein is selected from the group consisting of an interleukin,

an interferon, a chemokine, IL-2, IL-3, GM-CSF, B7-1, B7-2, IL-7, IL-12, IL-12 p35, IL-12 p40, IL-18, ICAM-1, LFA-3, and VCAM-1.

59. A replication-defective, recombinant adenoviral particle of claim 87 wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding an immunogen.

60. A replication-defective, recombinant adenoviral particle of claim 96 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

61. A replication-defective, recombinant adenoviral particle of claim 97 wherein said infectious agent is selected from the group consisting of a virus, bacteria, fungi, prion, mycoplasma and a parasite.

62. An isolated nucleic acid molecule of claim 98 wherien said virus is a human papilloma virus or a human immunodeficiency virus.

63. An isolated nucleic acid molecule of claim 97 wherein said immunogen is the human papilloma virus E6 protein or the human papilloma virus E7 protein.

64. A replication-defective, recombinant adenoviral particle of claim 97 wherein said cancer antigen is selected from the group consisting of MAGE, MUC1, MUC-2, CEA, MART, SP100, HER-2/NEU, PSA, tyrosinase, Melan-A, RAS, Rb, WT1, NF1, APC, DCC, and p53.

65. A replication-defective, recombinant adenoviral particle of claim 97 wherein said receptor is associated with autoimmune disease.

66. A replication-defective, recombinant adenoviral particle of claim 97 wherein said ligand is Beta Human Chorionic Gonadotropin.

67. An isolated nucleic acid molecule for generating immunity in an organism comprising an adenoviral inverted terminal repeat (ITR) fusion sequence, a packaging signal, a transcriptional control region operably linked to DNA encoding at least one immunogen, and an adeno-associated virus inverted terminal repeat (AAV-ITR), all operatively associated for generating an infectious, replication-defective recombinant adenoviral vector wherein the remaining portion of said nucleic acid molecule does not encode an adenoviral protein.

68. An isolated nucleic acid molecule of claim 104 wherein said adenoviral ITR fusion sequence comprises the Adenovirus type 5 (Ad5) right ITR and the Ad5 left inverted terminal repeat.

69. An isolated nucleic acid molecule of claim 105 wherein said ITR fusion sequence comprises Ad5 map unit 98.7 to map unit 1.26.

70. An isolated nucleic acid molecule of claim 105 wherein said ITR fusion sequence is the Eco47III (98.7 mu) to PvuII (1.26 mu) fragment of pBRX-AcsI.

71. An isolated nucleic acid molecule of claim 104 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

72. An isolated nucleic acid molecule of claim 105 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

73. An isolated nucleic acid molecule of claim 106 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

74. An isolated nucleic acid molecule of claim 107 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

75. An isolated nucleic acid molecule of claim 108 wherein said infectious agent is selected from the group consisting of a virus, bacteria, fungi, prion, mycoplasma and a parasite.

5

76. An isolated nucleic acid molecule of claim 112 wherein said virus is a human papilloma virus or a human immunodeficiency virus.

77. An isolated nucleic acid molecule of claim 111 wherein said immunogen is the

10 human papilloma virus E6 protein or the human papilloma virus E7 protein.

78. An isolated nucleic acid molecule of claim 111 wherein said cancer antigen is selected from the group consisting of MAGE, MUC1, MUC-2, CEA, MART, SP100, HER-2/NEU, PSA, tyrosinase, Melan-A, RAS, Rb, WT1, NF1, APC, DCC,

15 and p53.

79. An isolated nucleic acid molecule of claim 111 wherein said receptor is associated with autoimmune disease.

20 80. An isolated nucleic acid molecule of claim 111 wherein said ligand is Beta Human Chorionic Gonadotropin.

81. An isolated nucleic acid molecule of claim 104 wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding at least one immunogen

25 selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen; and a nucleic acid sequence encoding at least one immunomodulatory protein.

82. An isolated nucleic acid molecule of claim 118 wherein said immunomodulatory

30 protein is selected from the group consisting of an interleukin, an interferon, a chemokine, IL-2, IL-3, GM-CSF, B7-1, B7-2, IL-7, IL-12, IL-12 p35, IL-12 p40, IL18, ICAM-1, LFA-3, and VCAM-1.

83. A replication-defective, recombinant adenoviral particle for generating immunity in an organism comprising an adenoviral inverted terminal repeat (ITR) fusion sequence, a packaging signal, a transcriptional control region operably linked to DNA encoding at least one immunogen, and an adeno-associated virus inverted terminal repeat (AAV-ITR), all operatively associated for generating an infectious, replication-defective recombinant adenoviral vector wherein the remaining portion of said nucleic acid molecule does not encode an adenoviral protein.

84. A replication-defective, recombinant adenoviral particle of claim 120 wherein said adenoviral ITR fusion sequence comprises the Adenovirus type 5 (Ad5) right ITR and the Ad5 left inverted terminal repeat.

85. A replication-defective, recombinant adenoviral particle of claim 121 wherein said ITR fusion sequence comprises Ad5 map unit 98.7 to map unit 1.26.

86. A replication-defective, recombinant particle of claim 121 wherein said ITR fusion sequence is the Eco47III (98.7 mu) to PvuII (1.26 mu) fragment of pBRX-AcsI.

87. A replication-defective, recombinant adenoviral particle of claim 120 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

88. A replication-defective, recombinant adenoviral particle of claim 121 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

89. A replication-defective, recombinant adenoviral particle of claim 122 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

90. A replication-defective, recombinant adenoviral particle of claim 123 wherein said immunogen is selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein antigen, and a ligand antigen.

91. A replication-defective, recombinant adenoviral particle of claim 124 wherein said infectious agent is selected from the group consisting of a virus, bacteria, fungi, prion, mycoplasma and a parasite.

92. An isolated nucleic acid molecule of claim 128 wherein said virus is a human papilloma virus or a human immunodeficiency virus.

93. An isolated nucleic acid molecule of claim 127 wherein said immunogen is the human papilloma virus E6 protein or the human papilloma virus E7 protein.

94. A replication-defective, recombinant adenoviral particle of claim 127 wherein said cancer antigen is selected from the group consisting of MAGE, MUC1, MUC-2, CEA, MART, SP100, HER-2/NEU, PSA, tyrosinase, Melan-A, RAS, Rb, WT1, NF1, APC, DCC, and p53.

95. A replication-defective, recombinant adenoviral particle of claim 127 wherein said receptor is associated with autoimmune disease.

96. A replication-defective, recombinant adenoviral particle of claim 127 wherein said ligand is Beta Human Chorionic Gonadotropin.

97. A replication-defective, recombinant adenoviral particle of claim 120 wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding at least one immunogen selected from the group consisting of an infectious agent antigen, a cancer antigen, a receptor protein, and a ligand antigen; and a nucleic acid sequence encoding at least one immunomodulatory protein.

98. A replication-defective, recombinant adenoviral particle of claim 134 wherein said immunomodulatory protein is selected from the group consisting of an interleukin, an interferon, a chemokine, IL-2, IL-3, GM-CSF, B7-1, B7-2, IL-7, IL-12, IL-12 p35, IL-12 p40, IL18, ICAM-1, LFA-3, and VCAM-1.

99. A method for generating immunity in an organism comprising administering to the organism an adenoviral particle selected from the group consisting of an adenoviral particle of claim 54, claim 87, and claim 120.

The genome and transcription units of Ad5

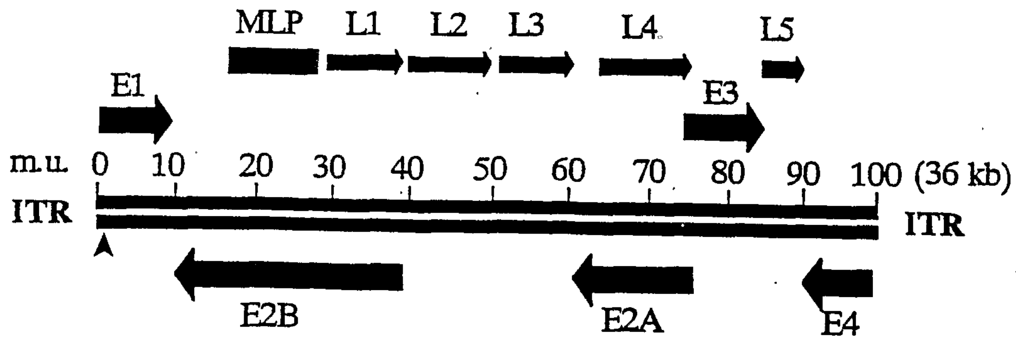


FIG. 1

Development of the 293 cell

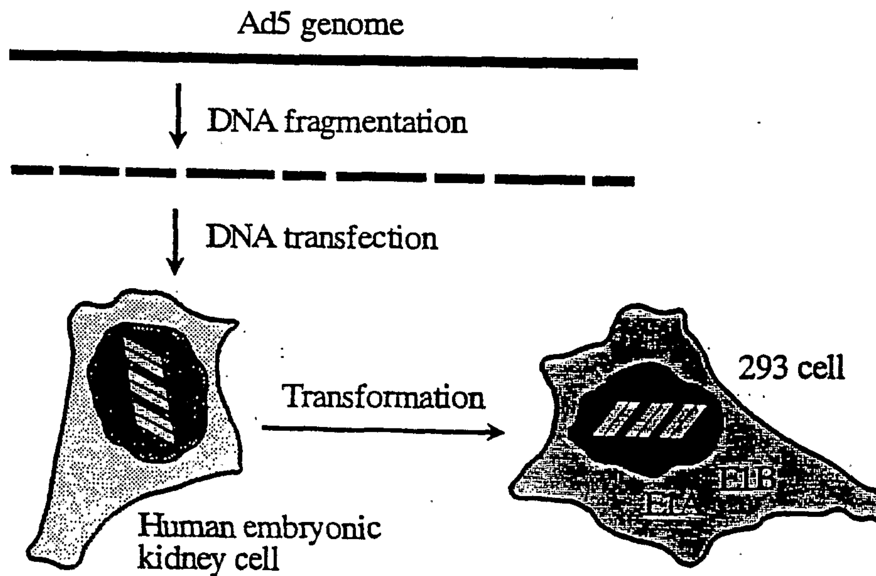
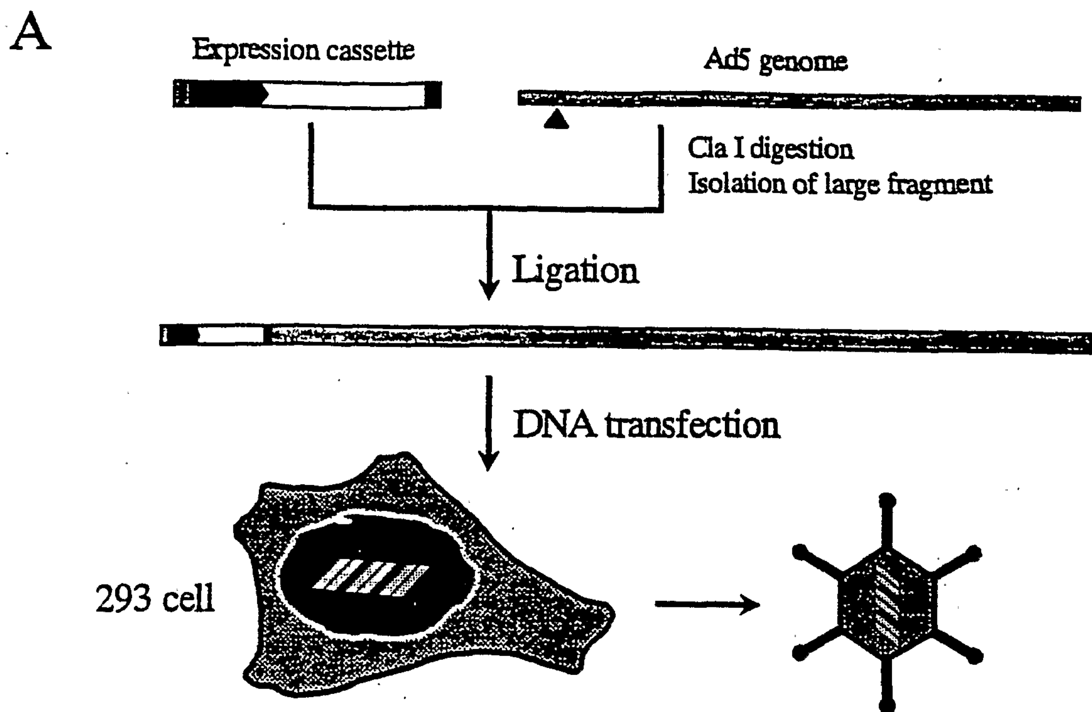


FIG. 2

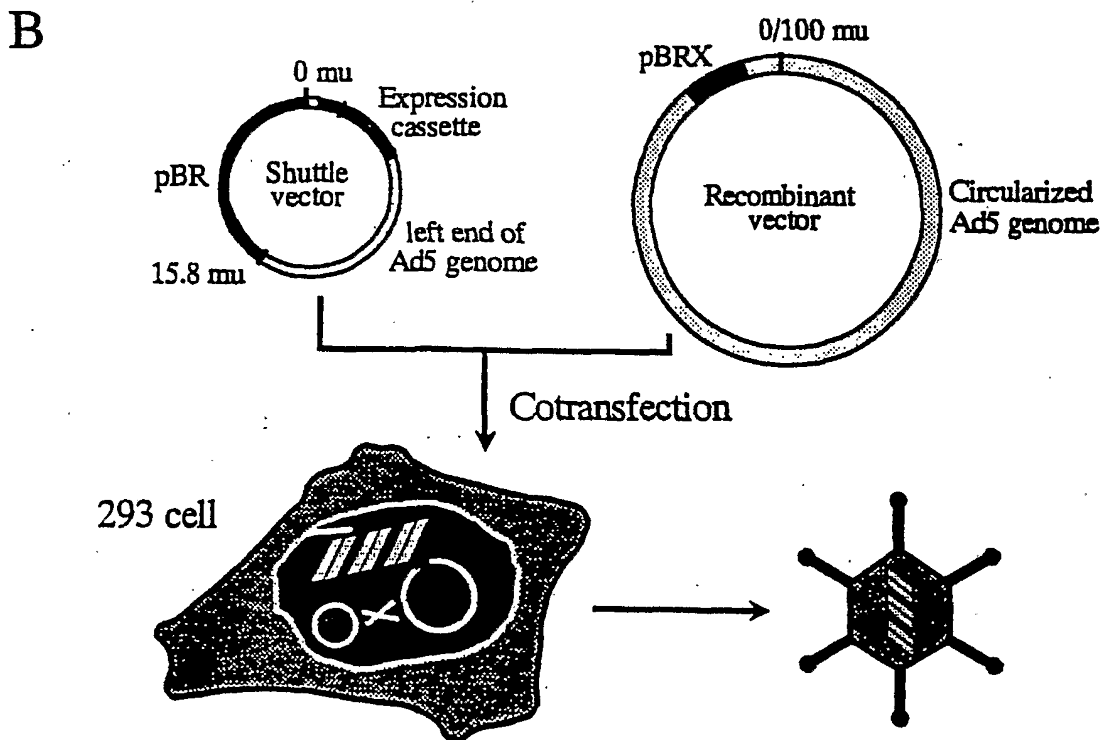
FIG. 3

Methods for generating recombinant Ad vectors

A



B



The Principle of The Mini-Ad Vector System

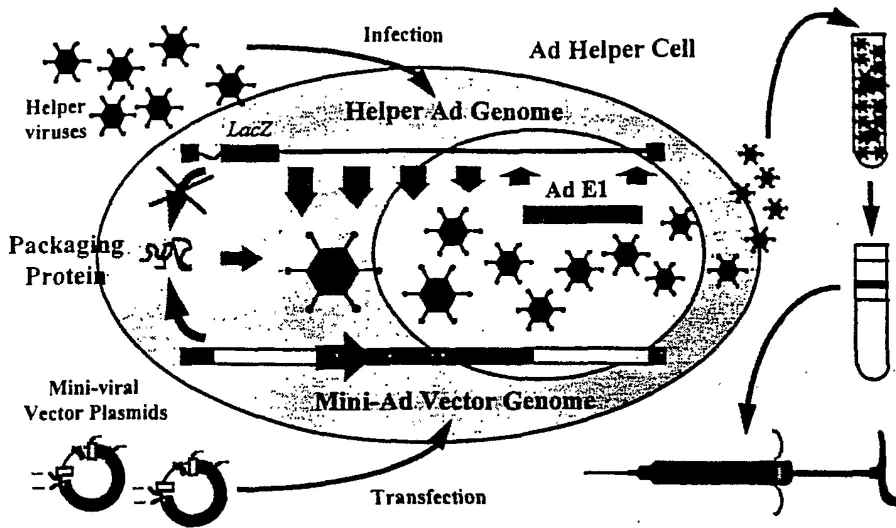


FIG. 4

Comparison of Available Ad Vector Systems

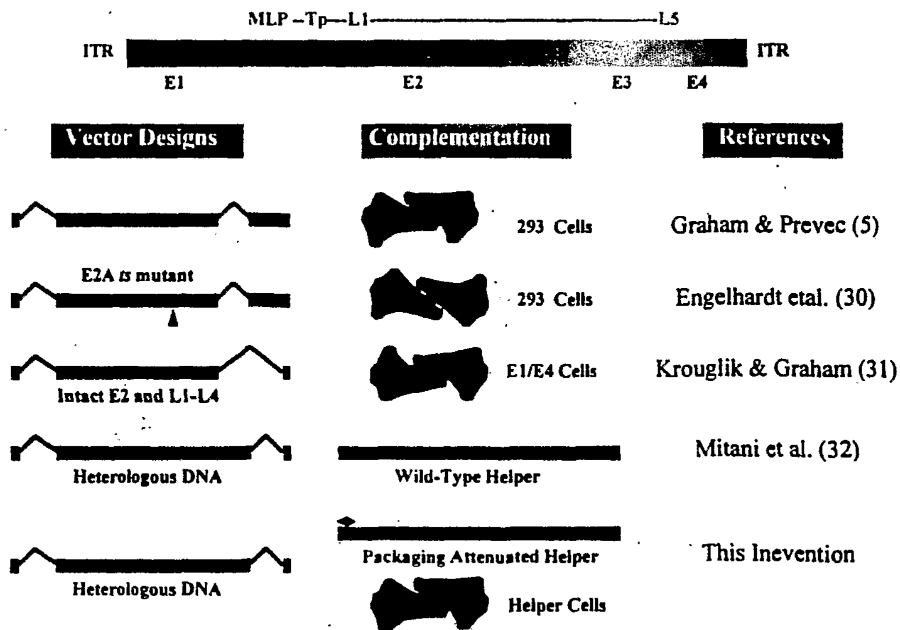
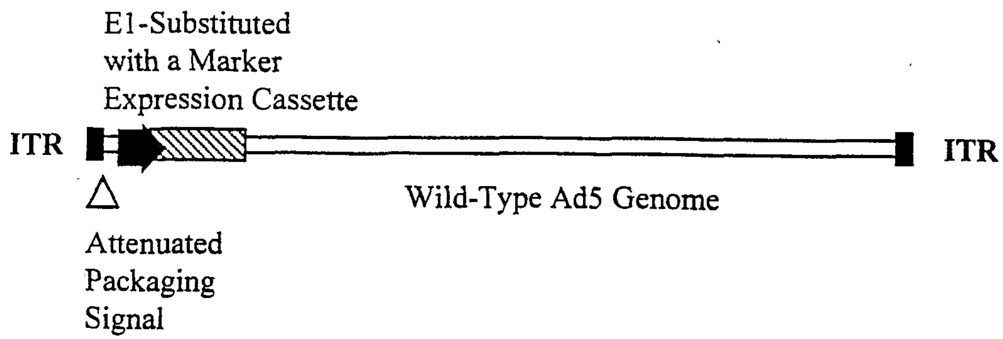


FIG. 5

FIG. 6

A.



B.

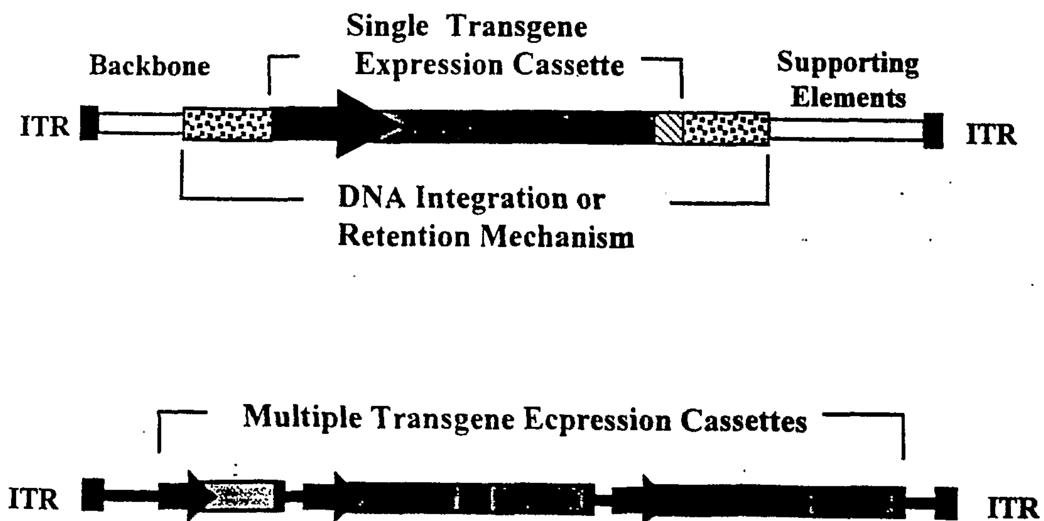
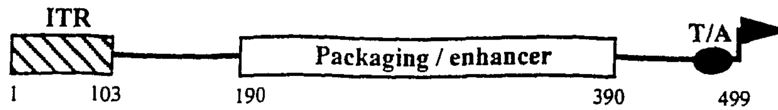


FIG. 7

A.



B.

```
     E1A Core               E2F                  E1A Core
GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG
CACATGTGTC CTTCACTGTT AAAAGCGCGC CAAAATCCGC CTACAACATC
        200        210        220        230        240

  A I                     A II             E2F
TAAATTTGGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG
ATTTAAACCC GCATTGGCTC ATTCTAAACC GGTAAAAGCG CCCTTTTGAC
        250        260        270        280        290

     E1A Core   A III      A IV
AATAAGAGGA AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGGGTAA
TTATTCTCCT TCACTTTAGA CTTATTAAAA CACAATGAGT ATCGCGCATT
        300        310        320        330        340

A V                   A VI       A VII
TATTTGTCTA GGGCCGCGGG GACTTTGACC GTTTACGTGG AGACTCGAAA
ATAAACAGAT CCCGGCGCCC CTGAAACTGG CAAATGCACC TCTGAGCGGG
        350        360        370        380        390
```

C.

A repeat	I	5'-GTAAATTTG -3'
A repeat	II	GTAAGATTTG
A repeat	III	GTCAAATCTG
A repeat	IV	ATAATTTTG
A repeat	V	GTAATATTTG
A repeat	VI	GGGACTTTG
A repeat	VII	GACCGTTTA

CONSENSUS: 5'- GT N_{3-4}TTTG -3'

FIG. 8

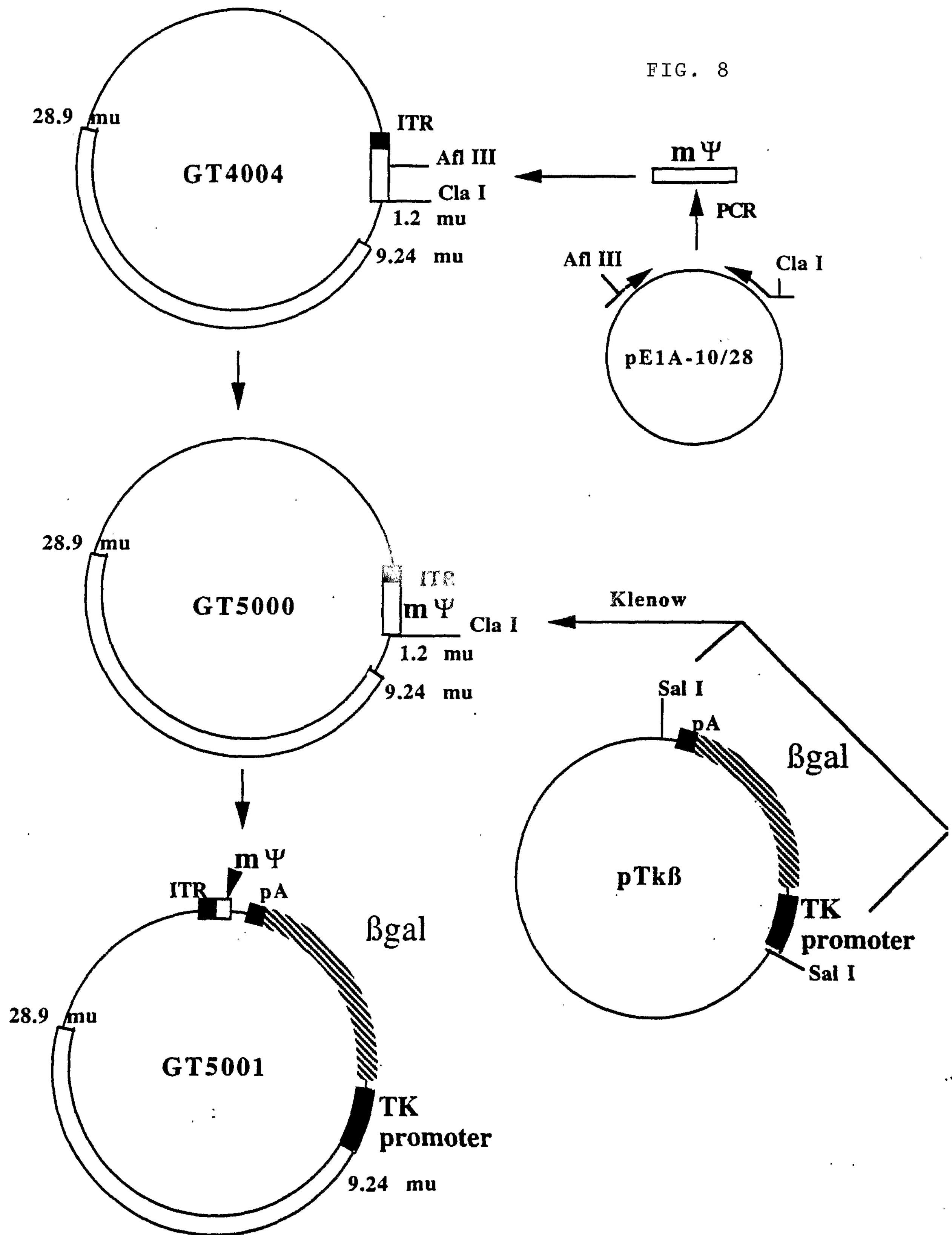


FIG. 9

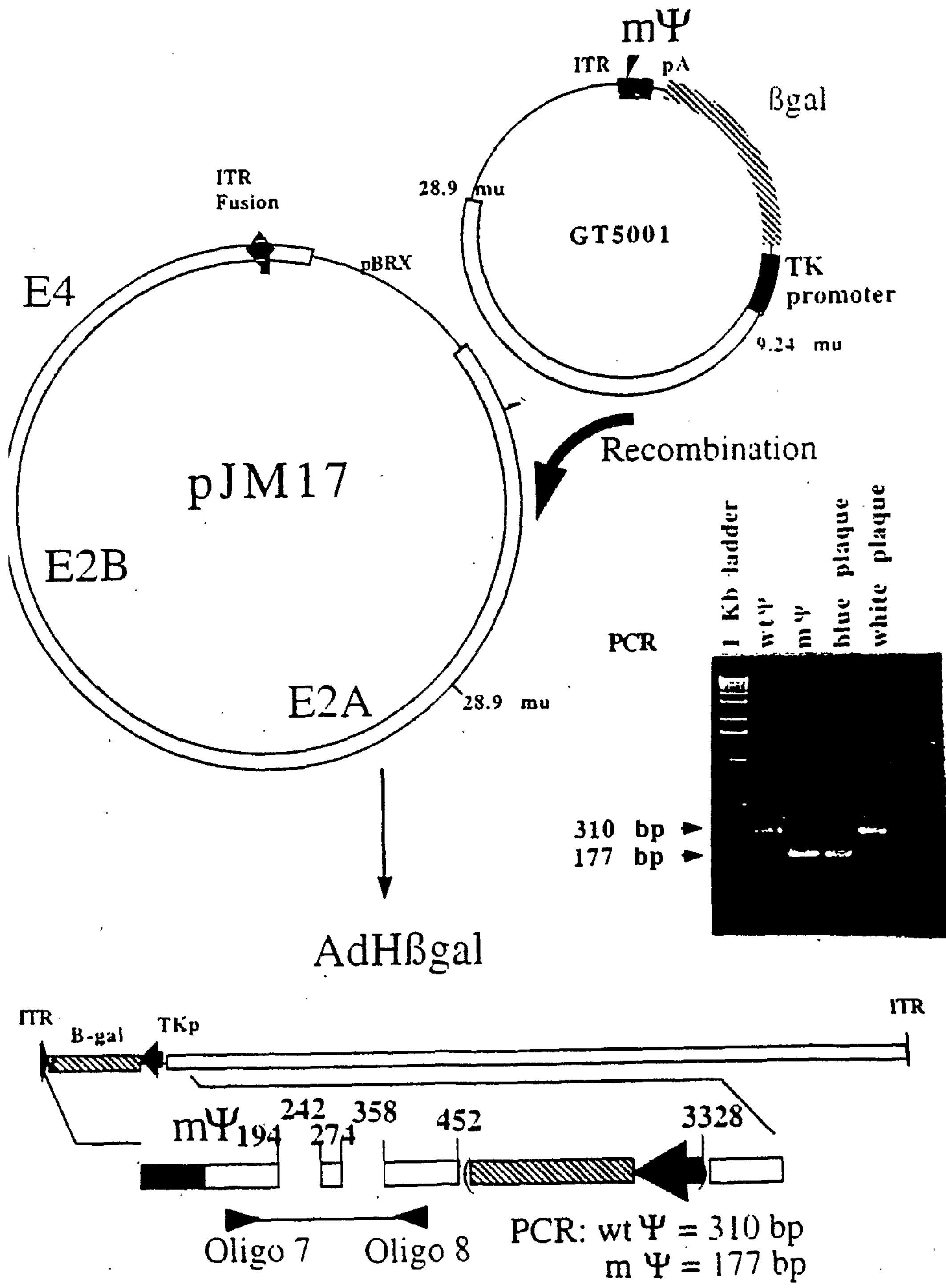


Fig 10

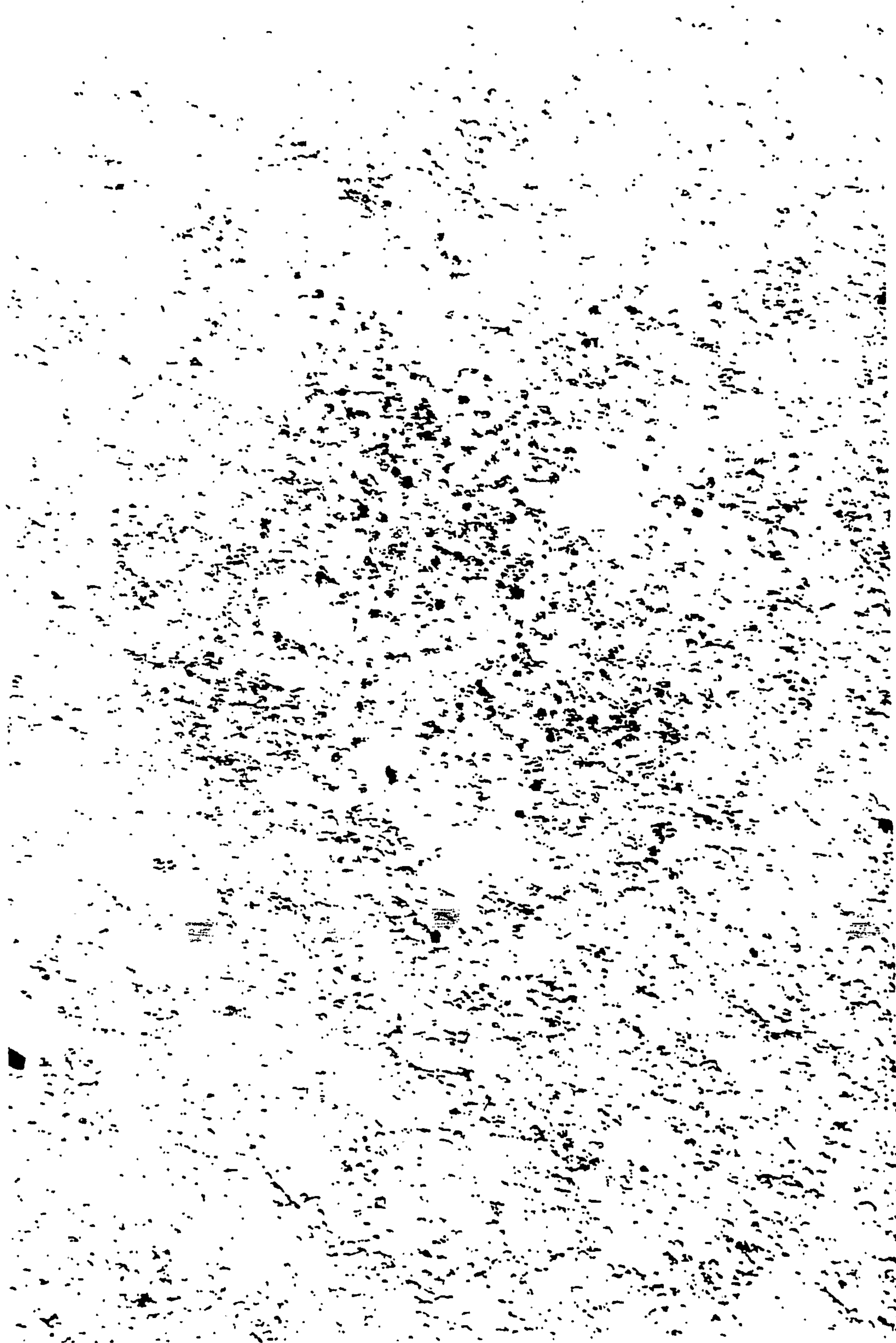


Fig 11.

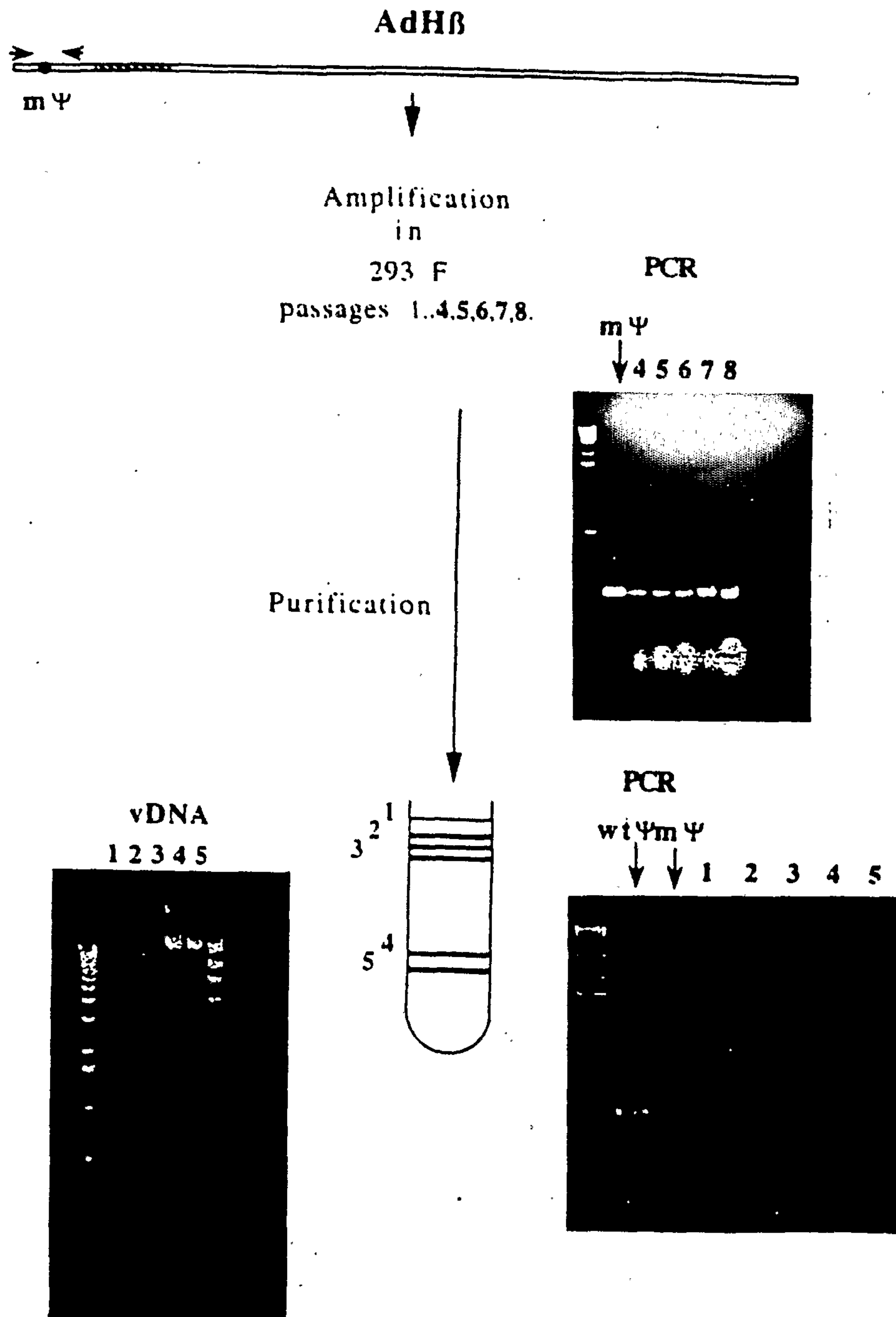


FIG. 12

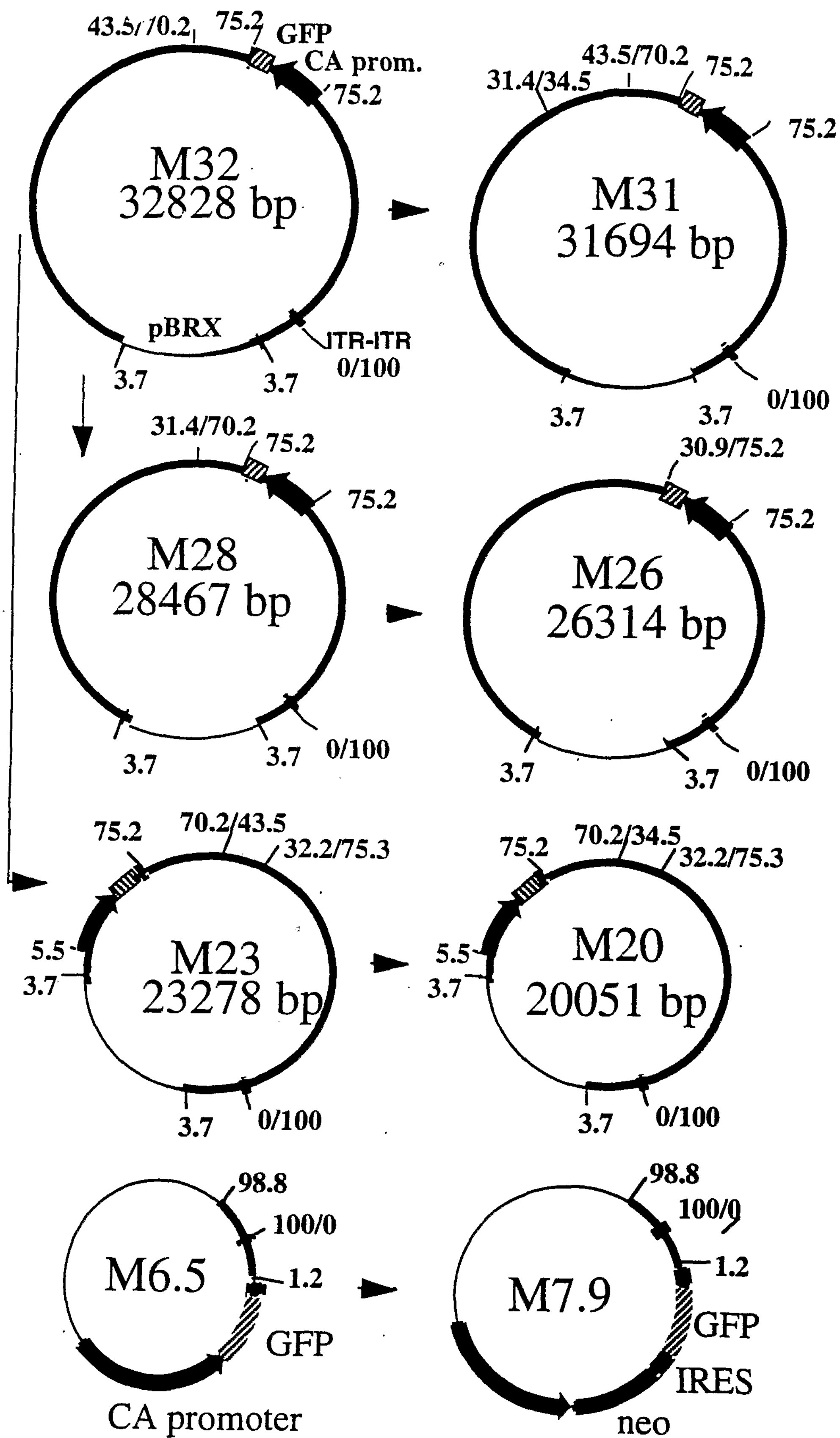
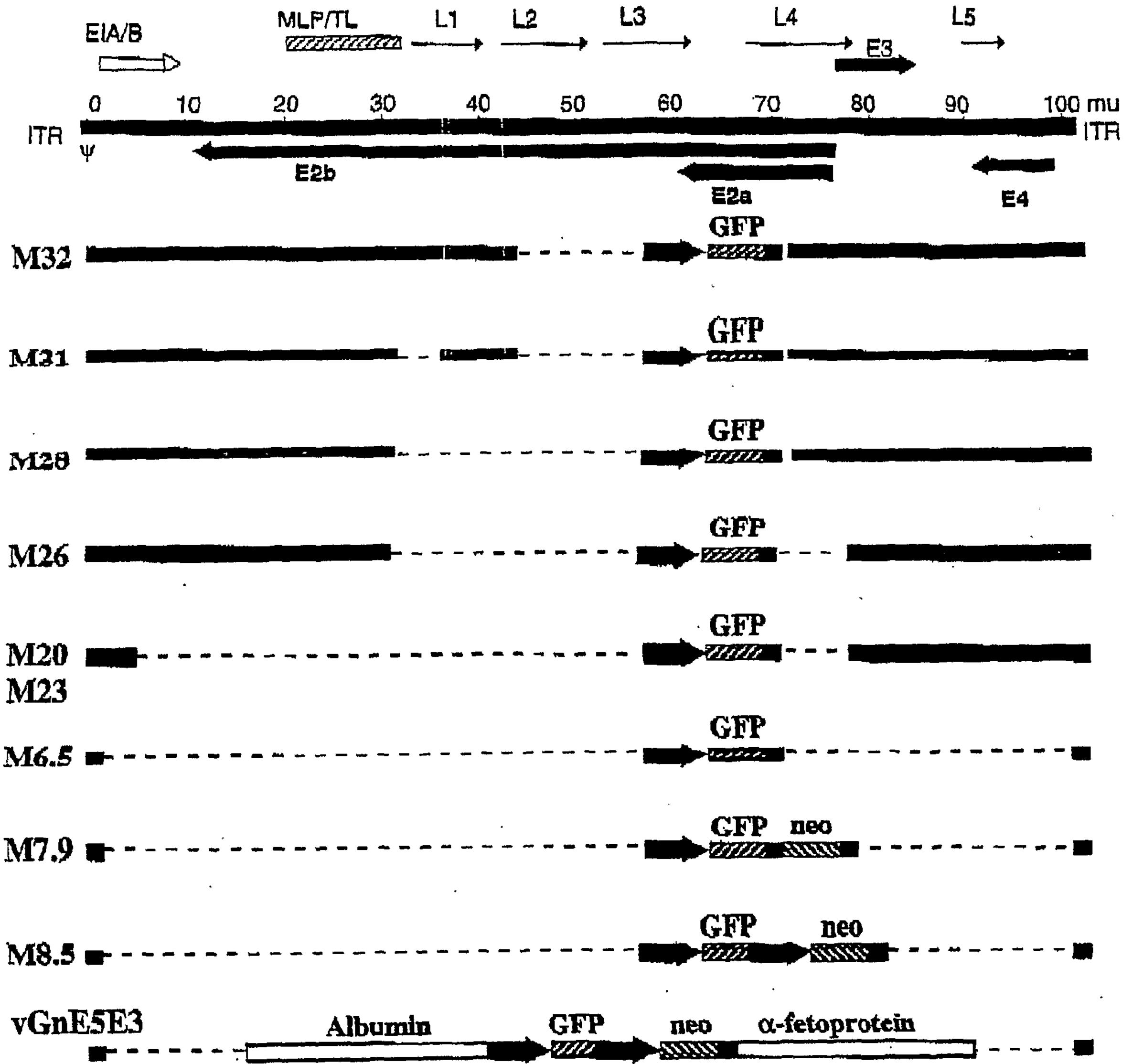


FIG. 13



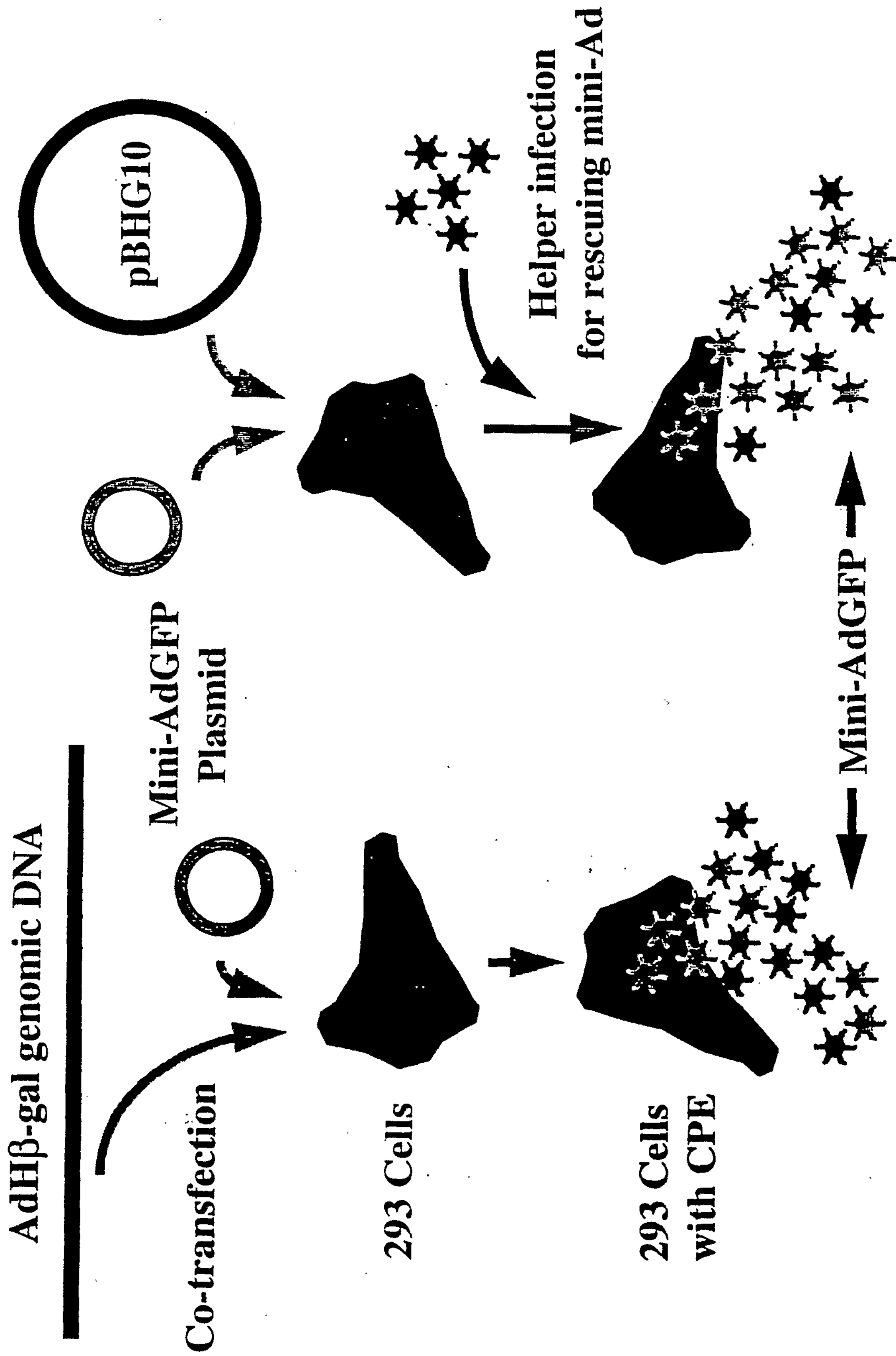


FIG. 14

Fig 5.

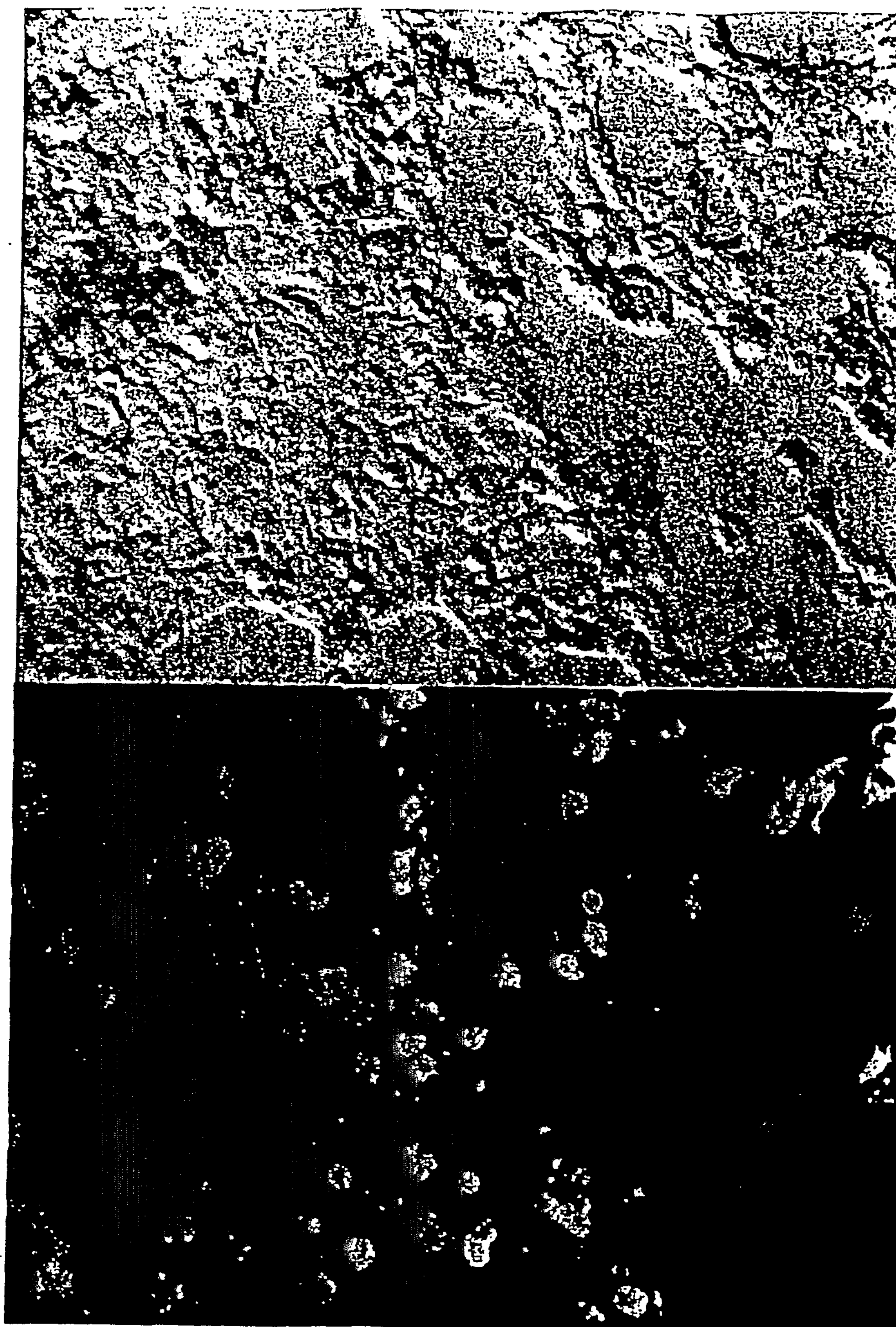
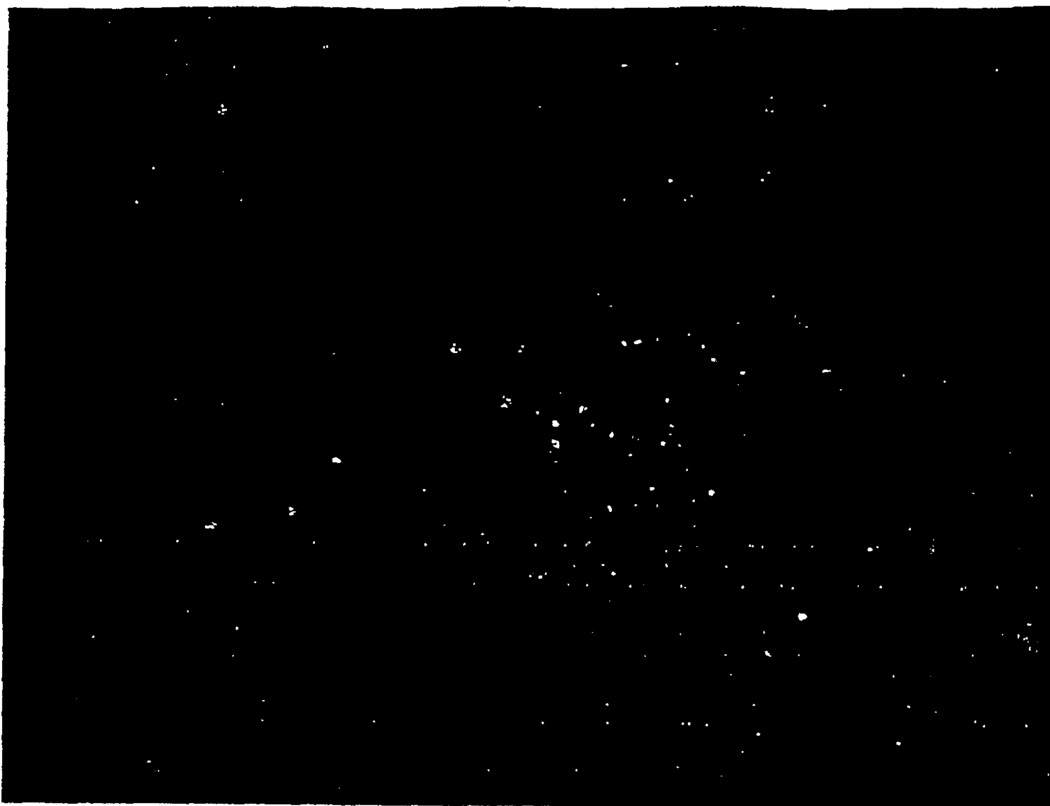
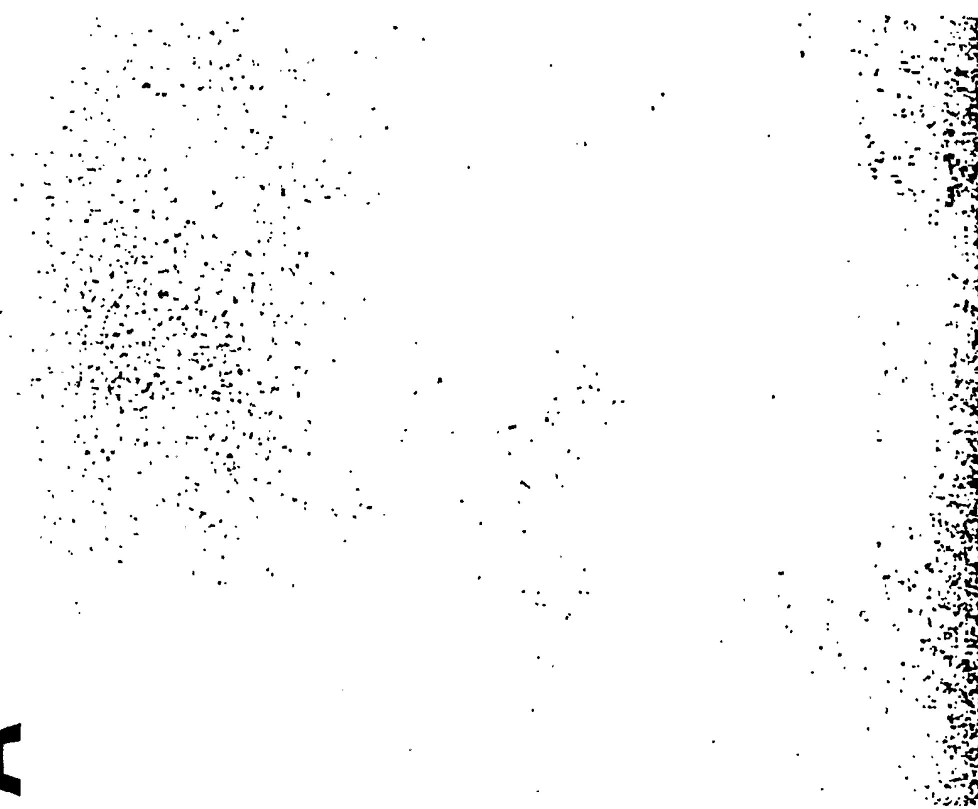


Fig 16





A

FIG. 17

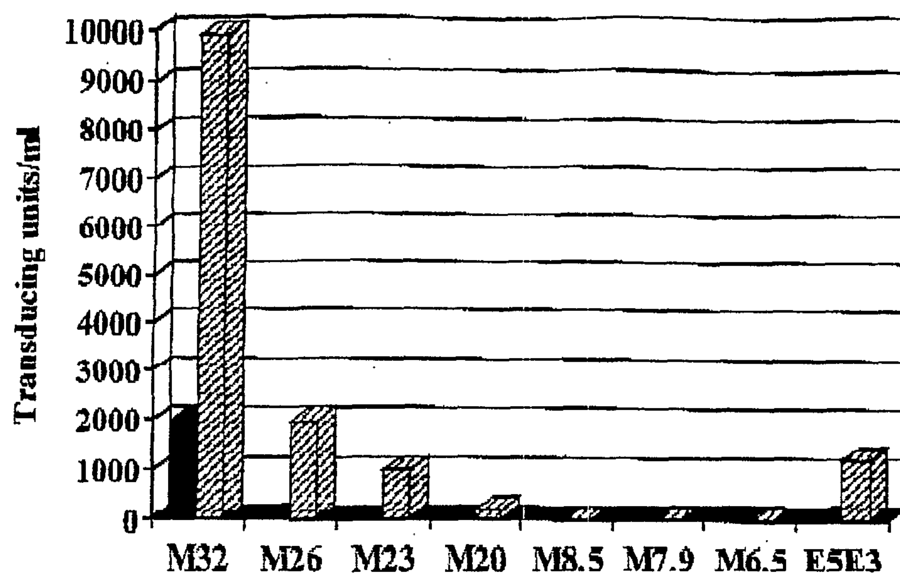
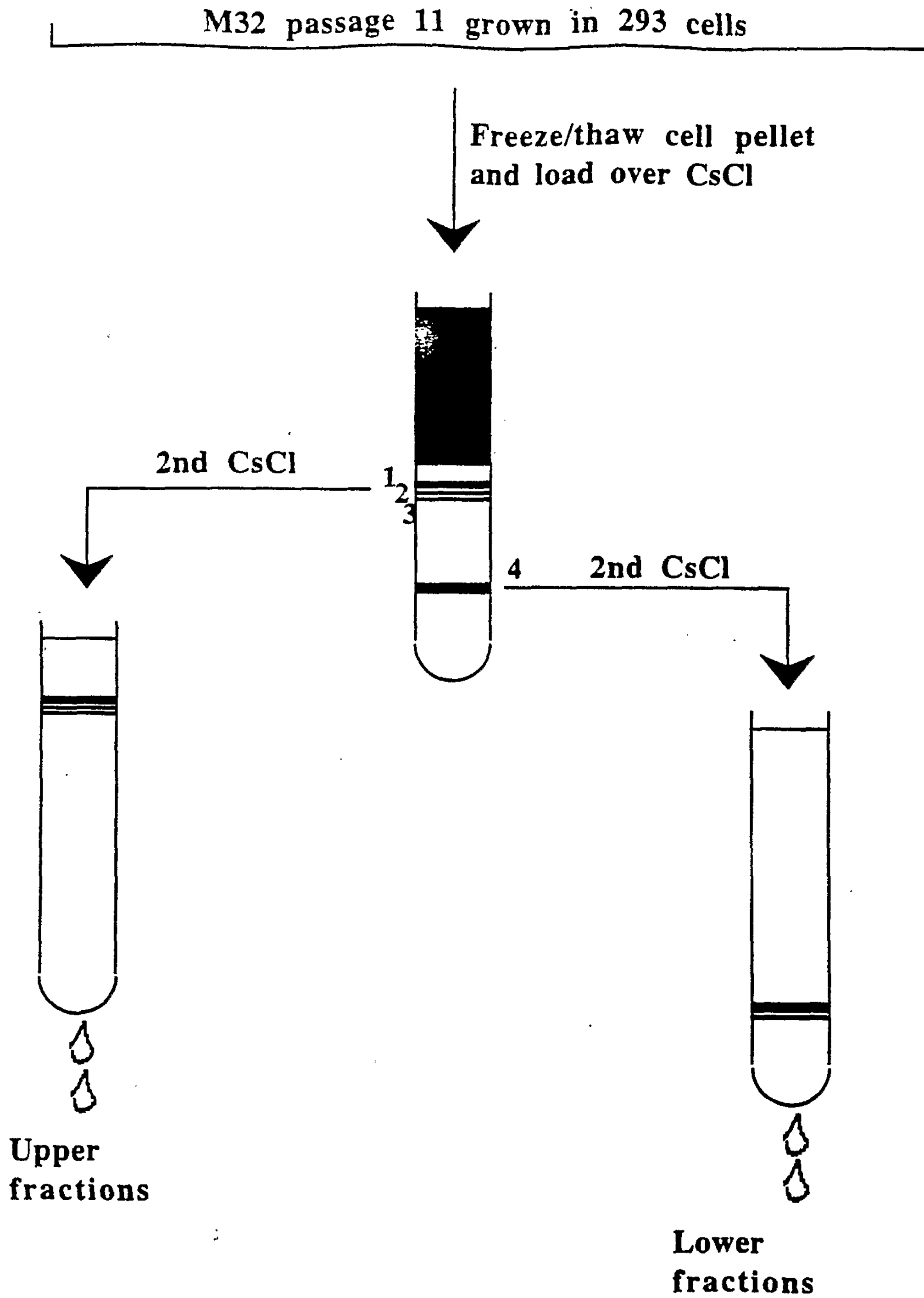


FIG. 18



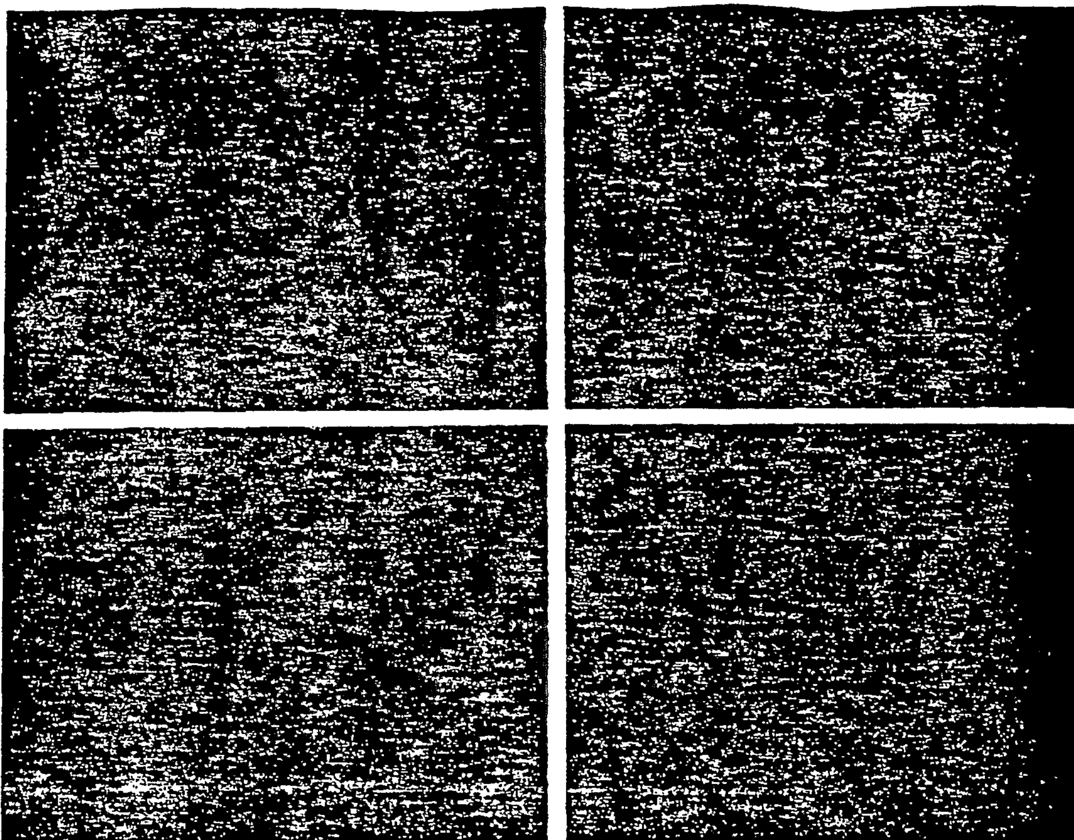
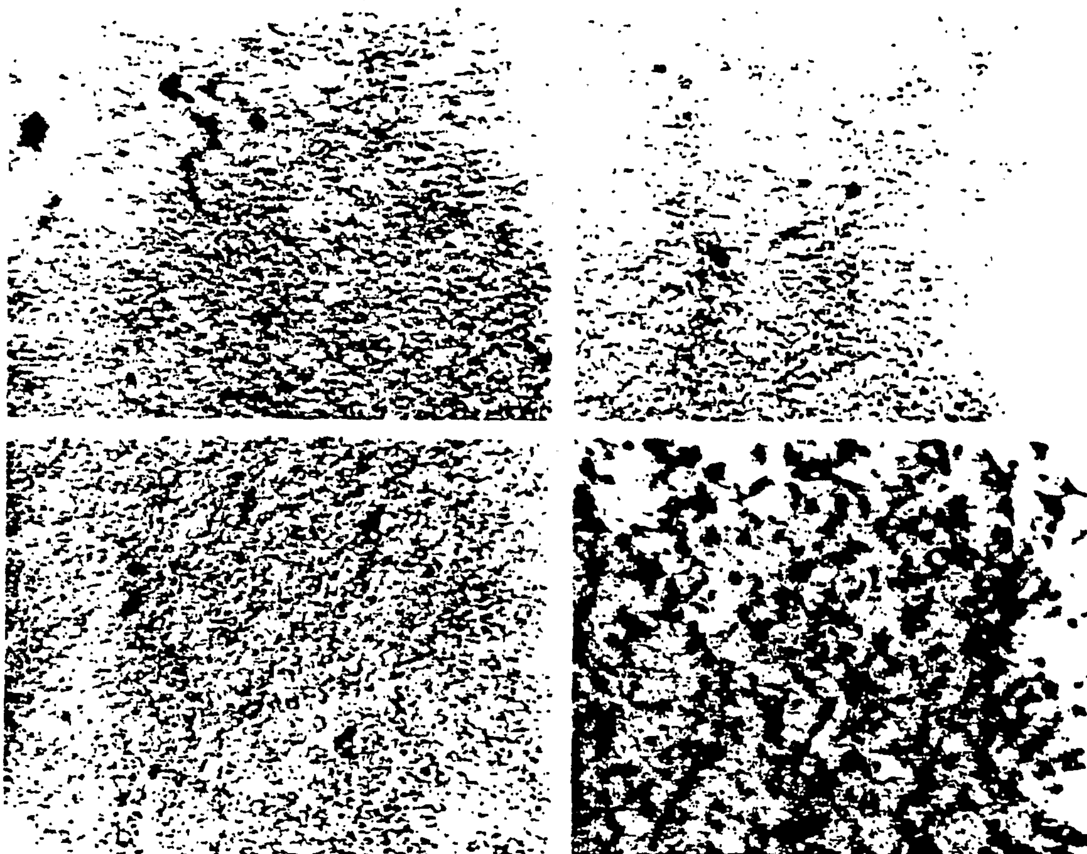


Fig 19



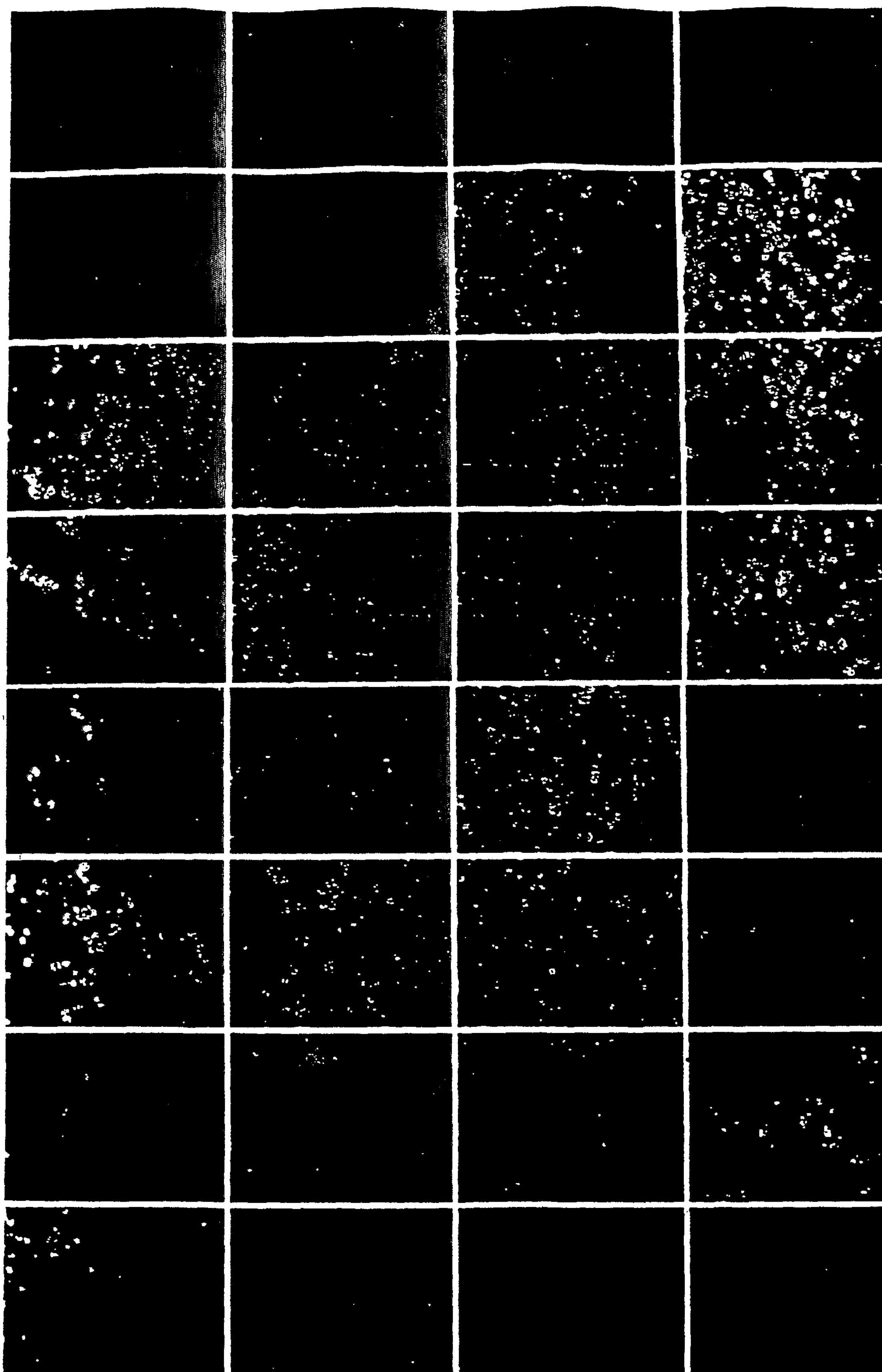


Fig 20A

Fig 20B

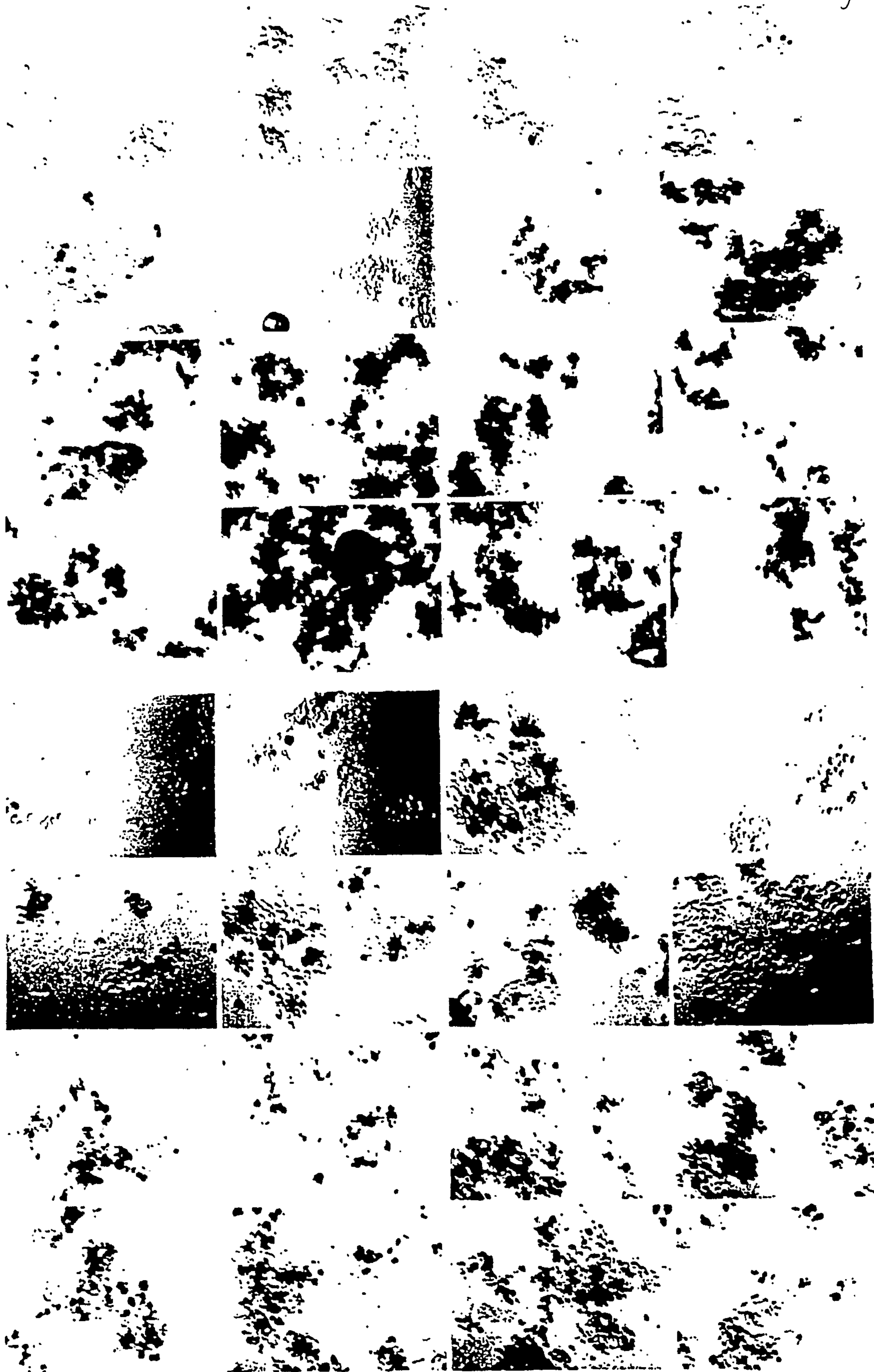
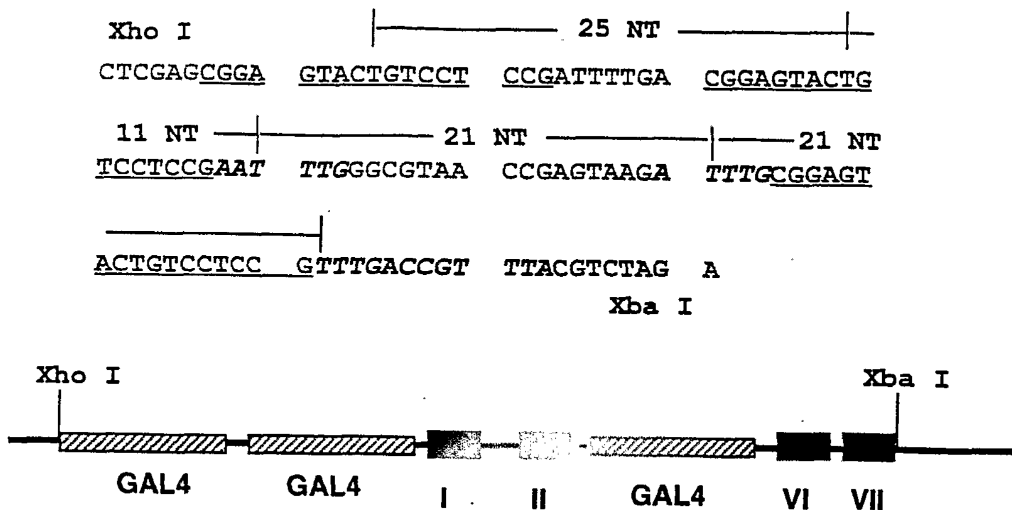


FIG. 21

Ad5 Pac Modification with GAL4 Binding Sites

Design #1



Design #2

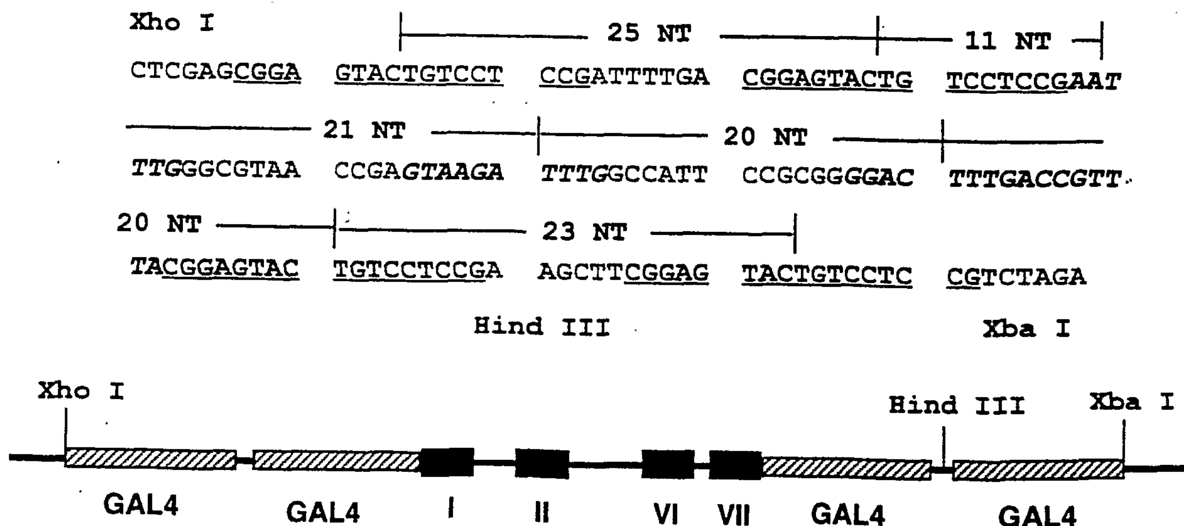
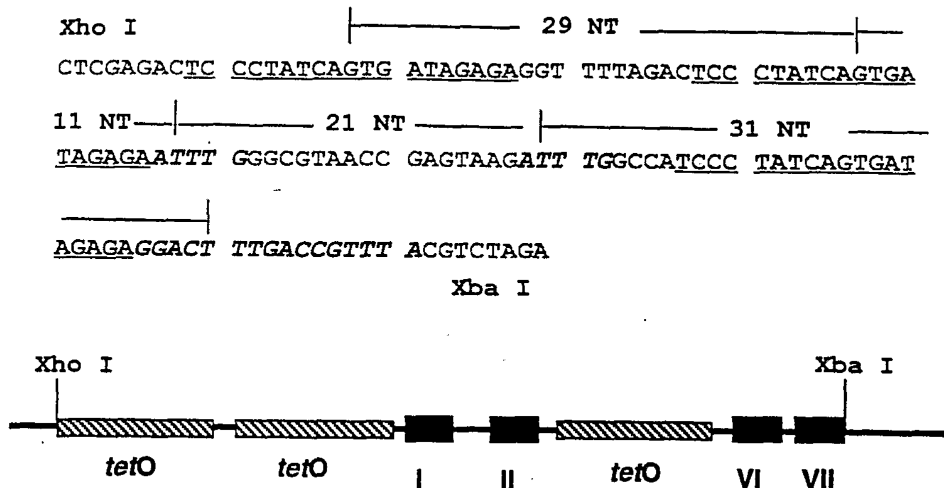


FIG. 22

Ad5 Pac Modification with *tet*O Sequences

Design #1



Design #2

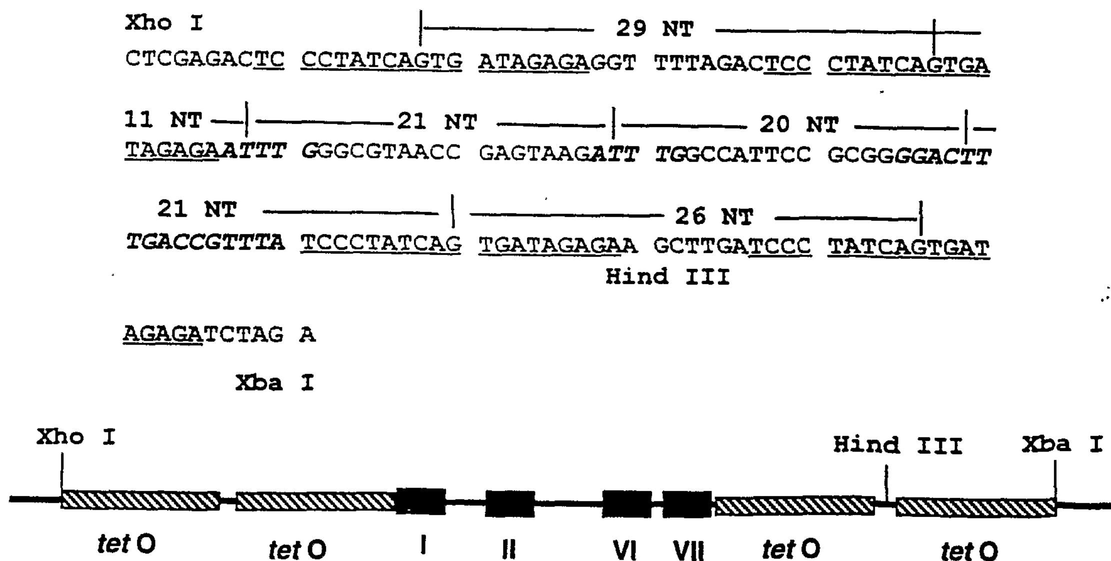
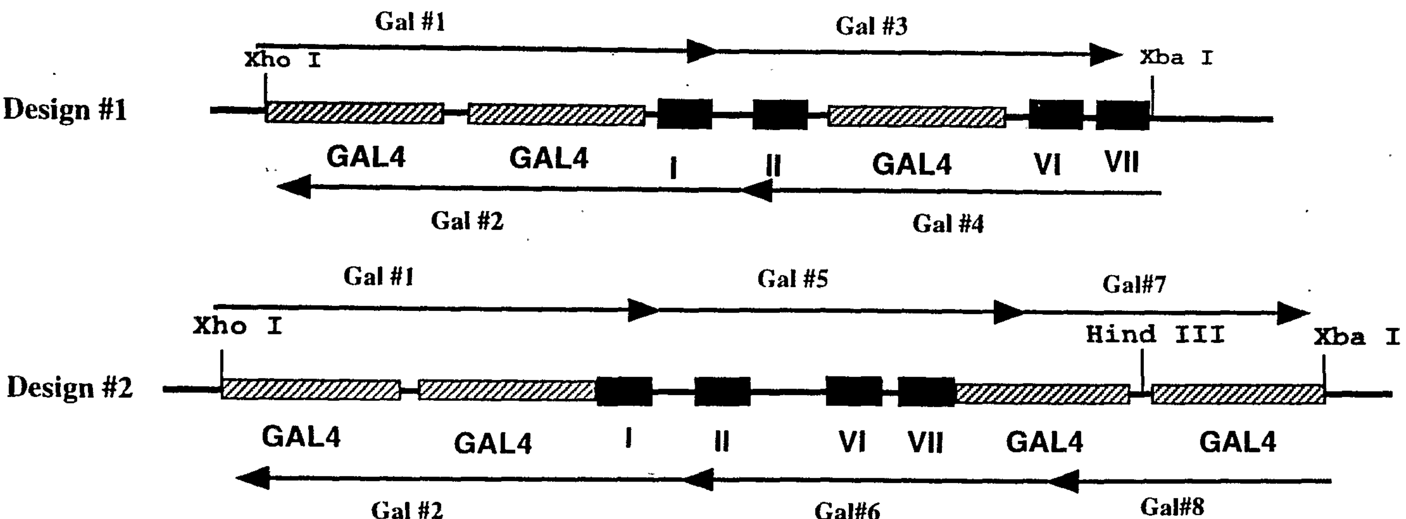


FIG. 23

Positions and Sequences of Synthetic Oligos for Ad Pac-GAL4 Modification (Design #1 & #2)



Gal#1 51 mer 5′ TCG AGC GGA GTA CTG TCC TCC GAT TTT GAC GGA GTA CTG TCC TCC GAA TTT 3′

Gal#2 53 mer 5′ ACG CCC AAA TTC GGA GGA CAG TAC TCC GTC AAA ATC GGA GGA CAG TAC TCC GC 3′

Gal#3 54 mer 5′ GGG CGT AAC CGA GTA AGA TTT GCG GAG TAC TGT CCT CCG TTT GAC CGT TTA CGT 3

Gal#4 52 mer 5′ CTA GAC GTA AAC GGT CAA ACG GAG GAC AGT ACT CCG CAA ATC TTA CTC GGT T 3′

Gal#5 42 mer 5′ GGG CGT AAC CGA GTA AGA TTT GGC CAT TCC GCG GGG ACT TTG 3′

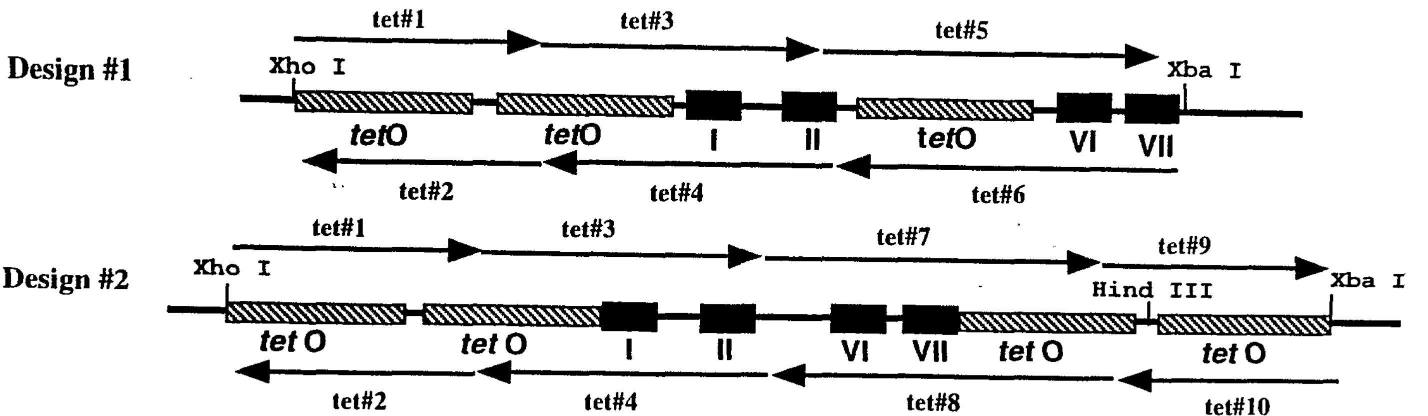
Gal#6 42 mer 5′ AAC GGT CAA AGT CCC CGC GGA ATG GCC AAA TCT TAC TCG GTT 3′

Gal#7 49 mer 5′ ACC GTT TAC GGA GTA CTG TCC TCC GAA GCT TCG GAG TAC TGT CCT CCG T 3′

Gal#8 47 mer 5′ CTA GAC GGA GGA CAG TAC TCC GAA GCT TCG GAG GAC AGT ACT CCG TA 3′

FIG. 24

Positions and Sequences of Synthetic Oligos for Ad Pac-*tet*O Modification (Design #1 & #2)



tet#1 40 mer 5′ TCG AGA CTC CCT ATC AGT GAT AGA GAG GTT TTA GAC TCC C 3′

tet#2 42 mer 5′ CTG ATA GGG AGT CTA AAA CCT CTC TAT CAC TGA TAG GGA GTC 3′

tet#3 36 mer 5′ TAT CAG TGA TAG AGA ATT TGG GCG TAA CCG AGT AAG 3′

tet#4 36 mer 5′ CCA AAT CTT ACT CGG TTA CGC CCA AAT TCT CTA TCA 3′

tet#5 47 mer 5′ ATT TGG CCA TCC CTA TCA GTG ATA GAG AGG ACT TTG ACC GTT TAC GT 3′

tet#6 45 mer 5′ CTA GAC GTA AAC GGT CAA AGT CCT CTC TAT CAC TGA TAG GGA TGG 3′

tet#7 48 mer 5′ ATT TGG CCA TTC CGC GGG GAC TTT GAC CGT TTA TCC CTA TCA GTG ATA 3′

tet#8 48 mer 5′ CTT CTC TAT CAC TGA TAG GGA TAA ACG GTC AAA GTC CCC GCG GAA TGG 3′

tet#9 31 mer 5′ GAG AAG CTT GAT CCC TAT CAG TGA TAG AGA T 3′

tet#10 29 mer 5′ CTA GAT CTC TAT CAC TGA TAG GGA TCA AG 3′

FIG. 25

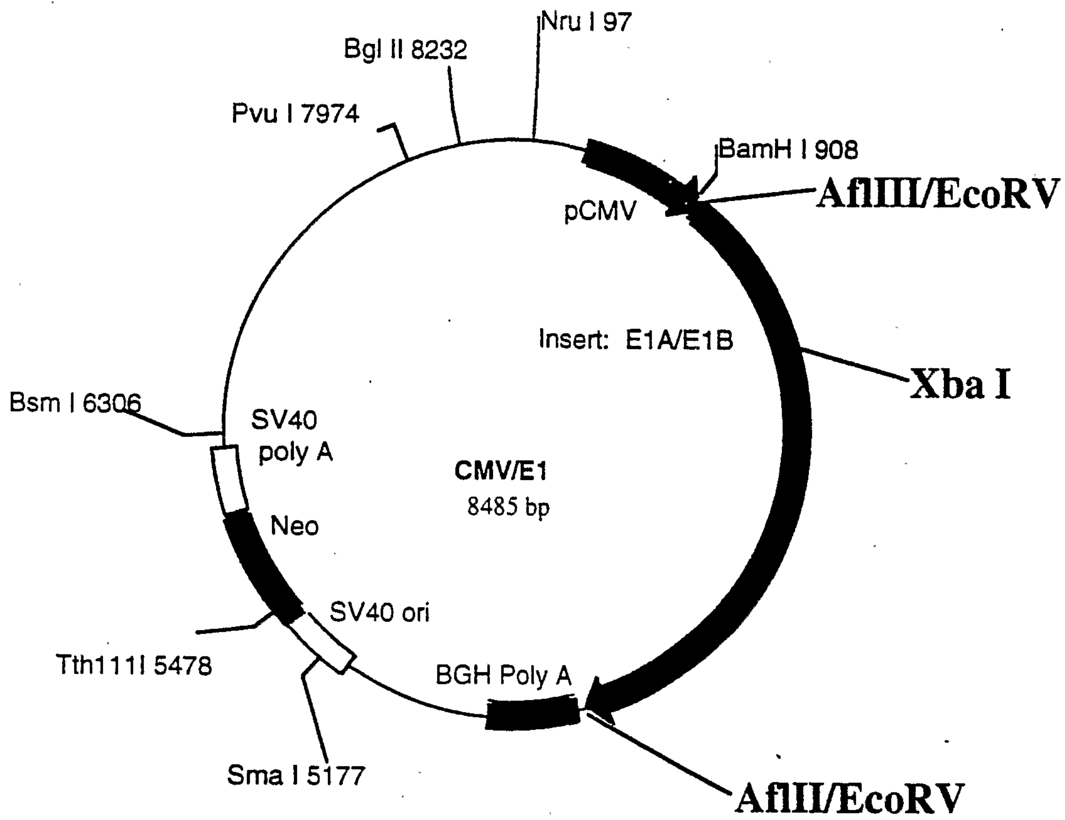


Fig 26

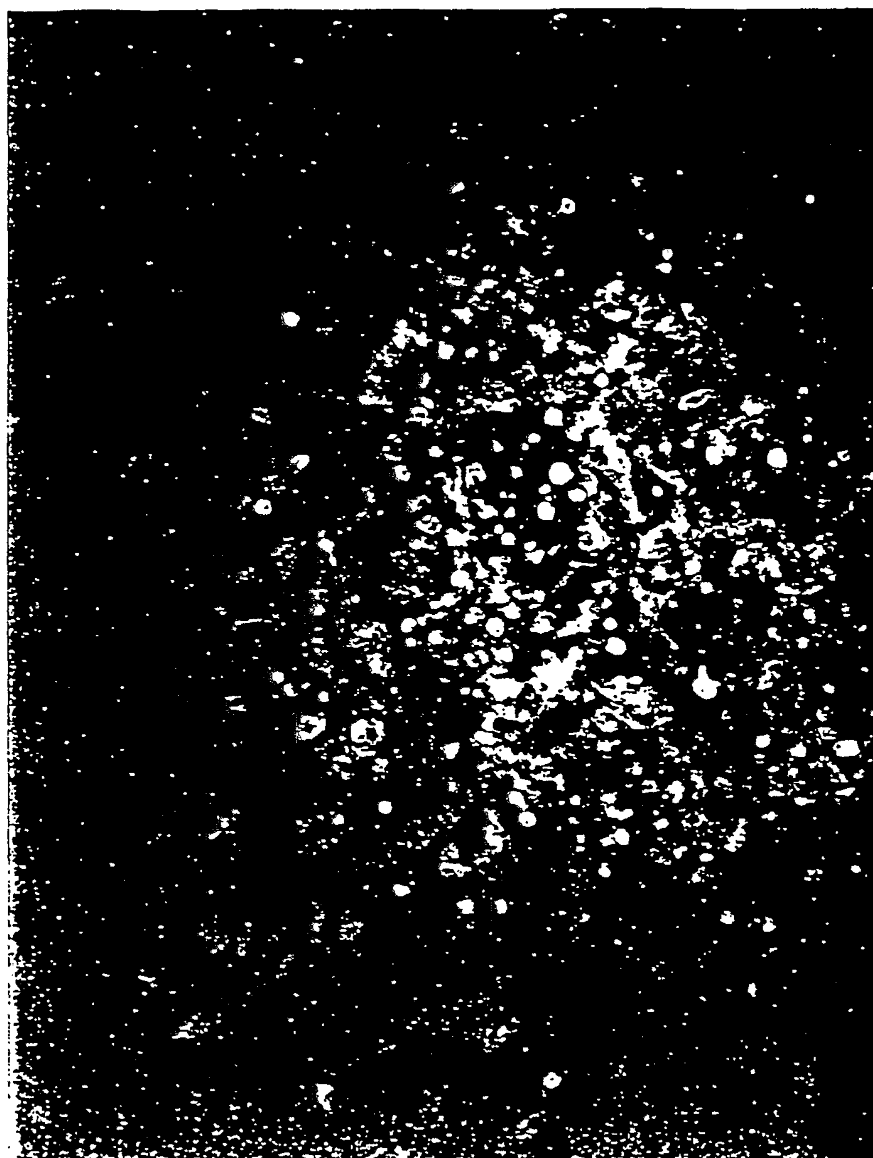


Fig 27.

E1 Cell Lines - Southern Blot Analysis

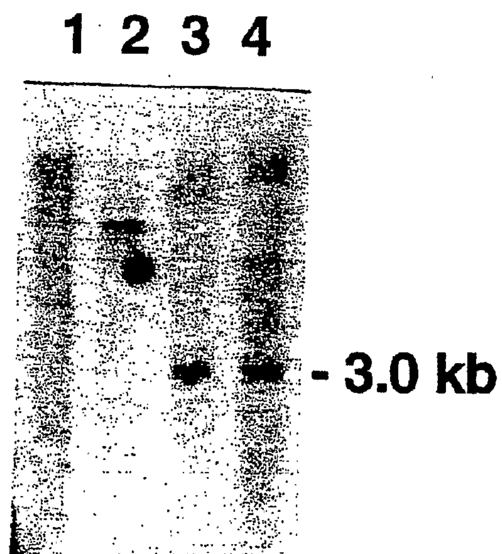
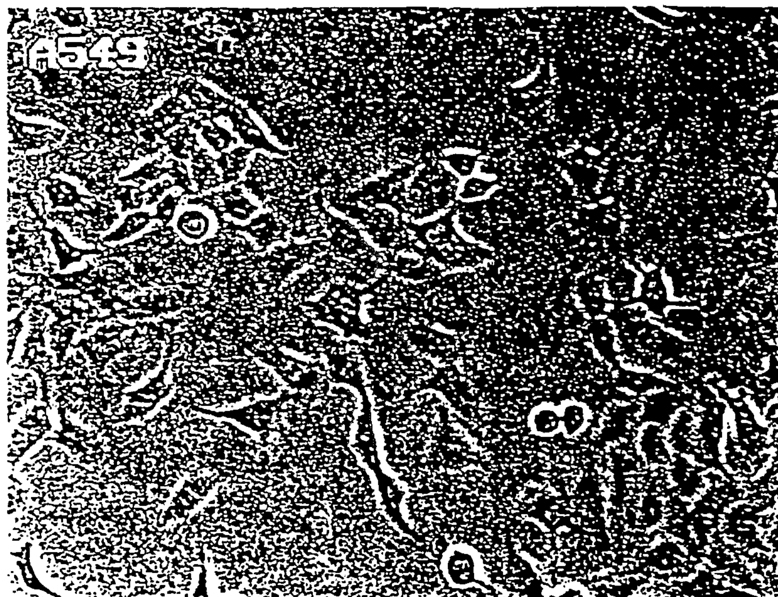
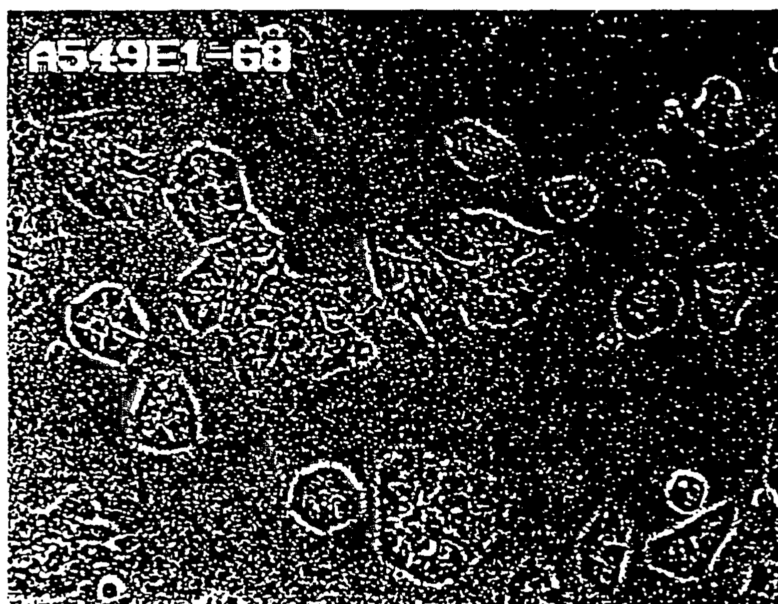


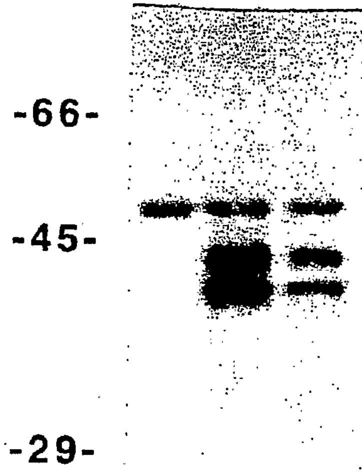
Fig 28.





Western Blot Analysis - E1A

Fig-9.



Western Blot Analysis - E1B

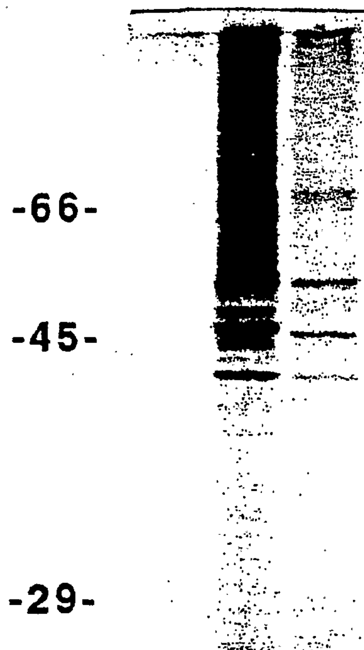


FIG. 30

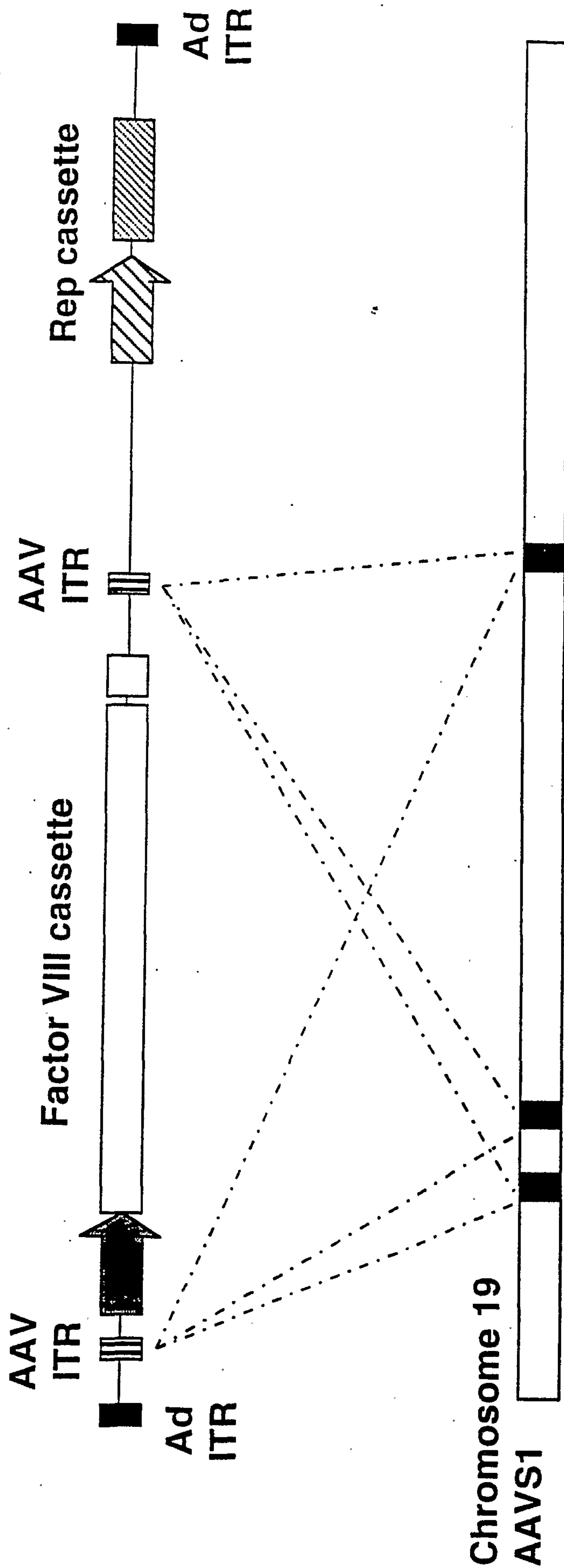
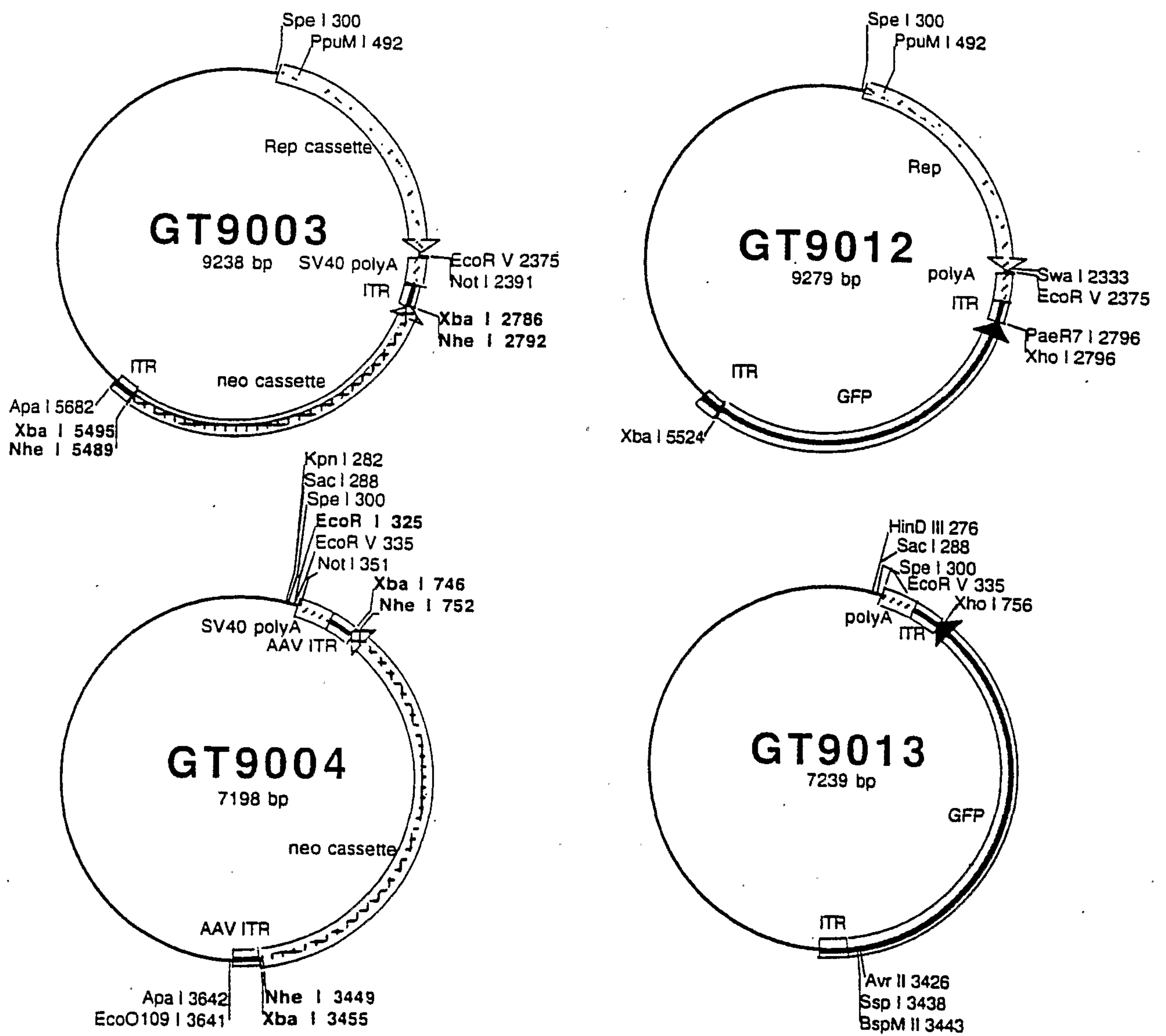


FIG. 31



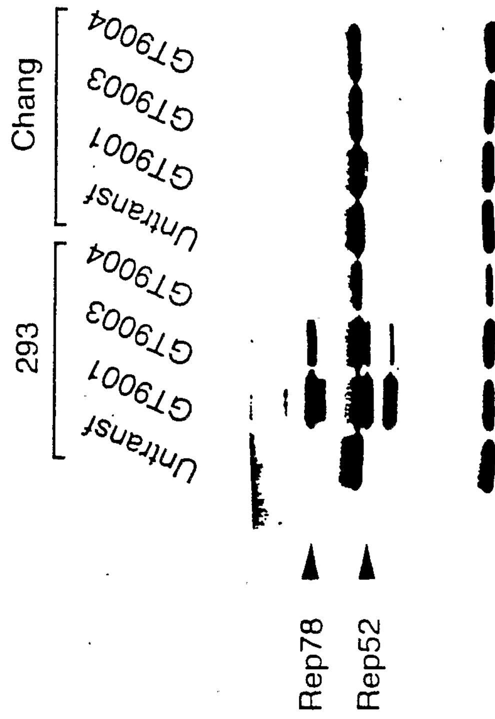
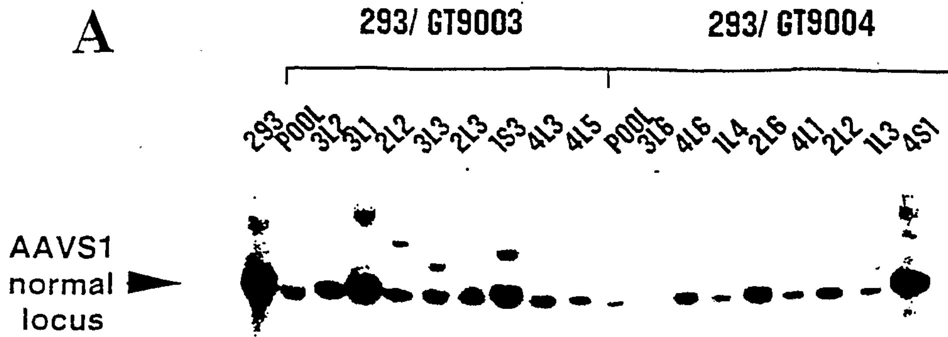

Chang
293
GT9004
GT9003
GT9001
Untransf
GT9004
GT9003
GT9001
Untransf
Rep78
Rep52

Fig 33



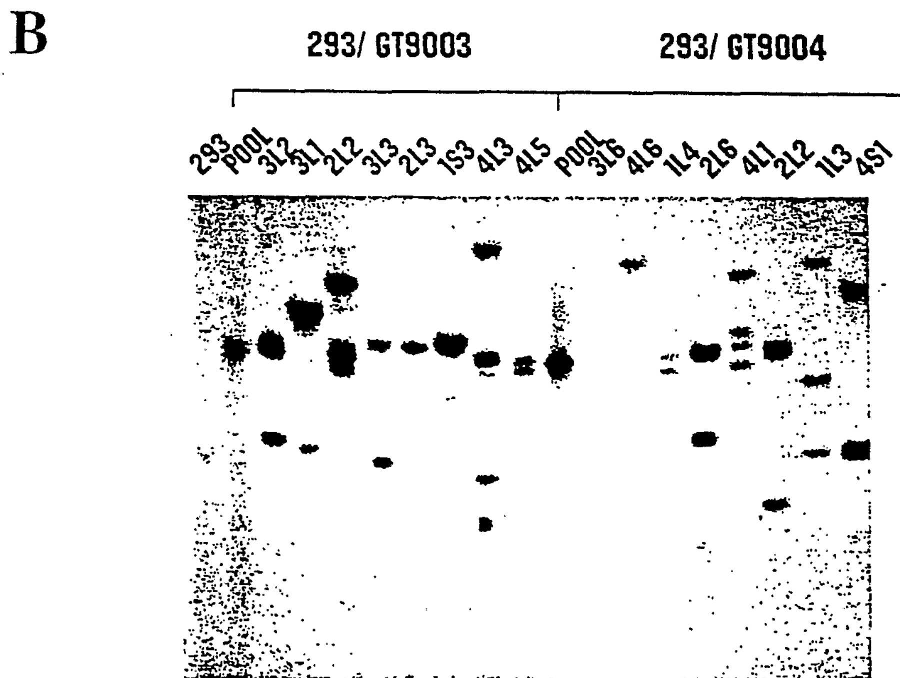
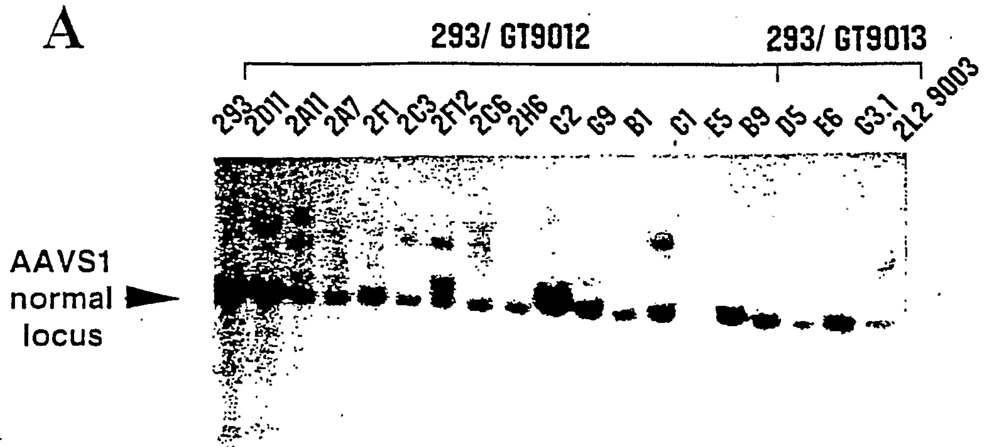


Fig 34

A



B

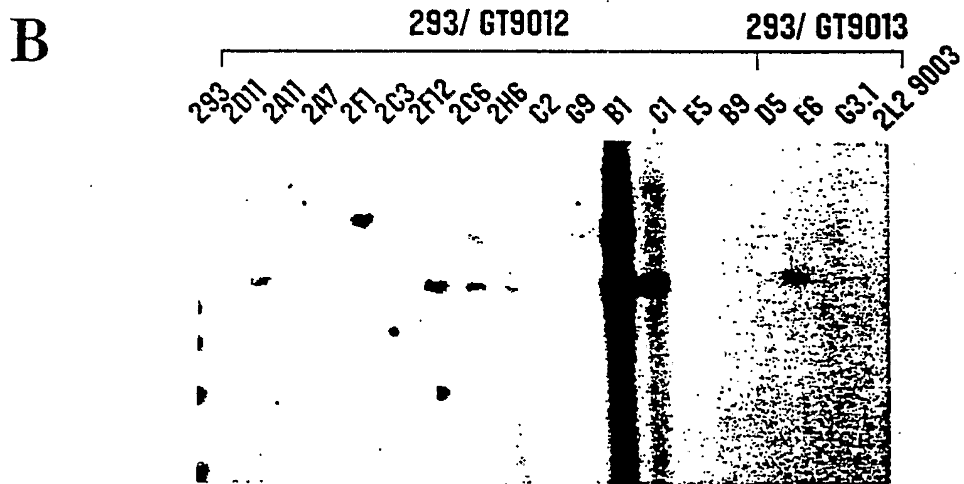


FIG. 35

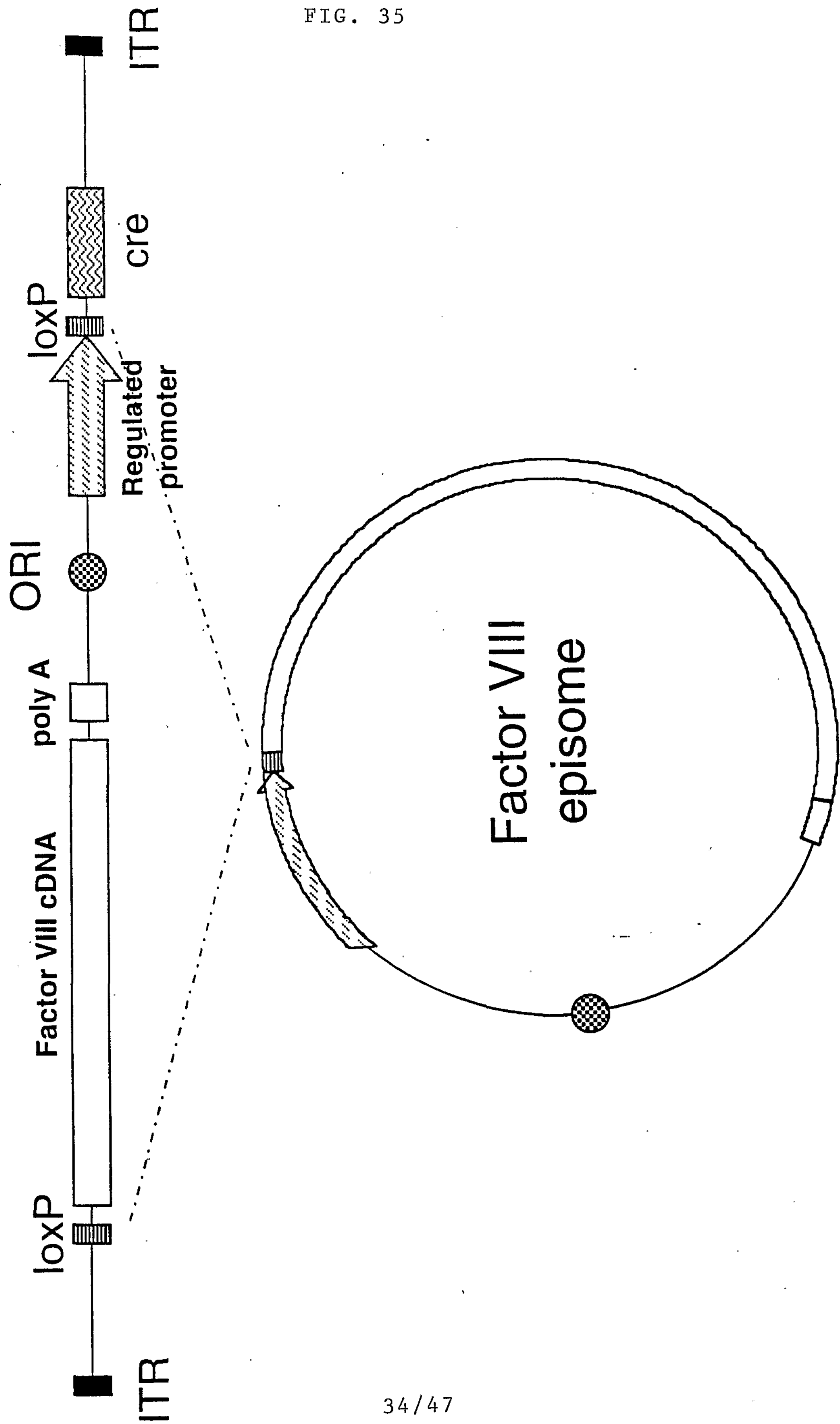


FIG. 36

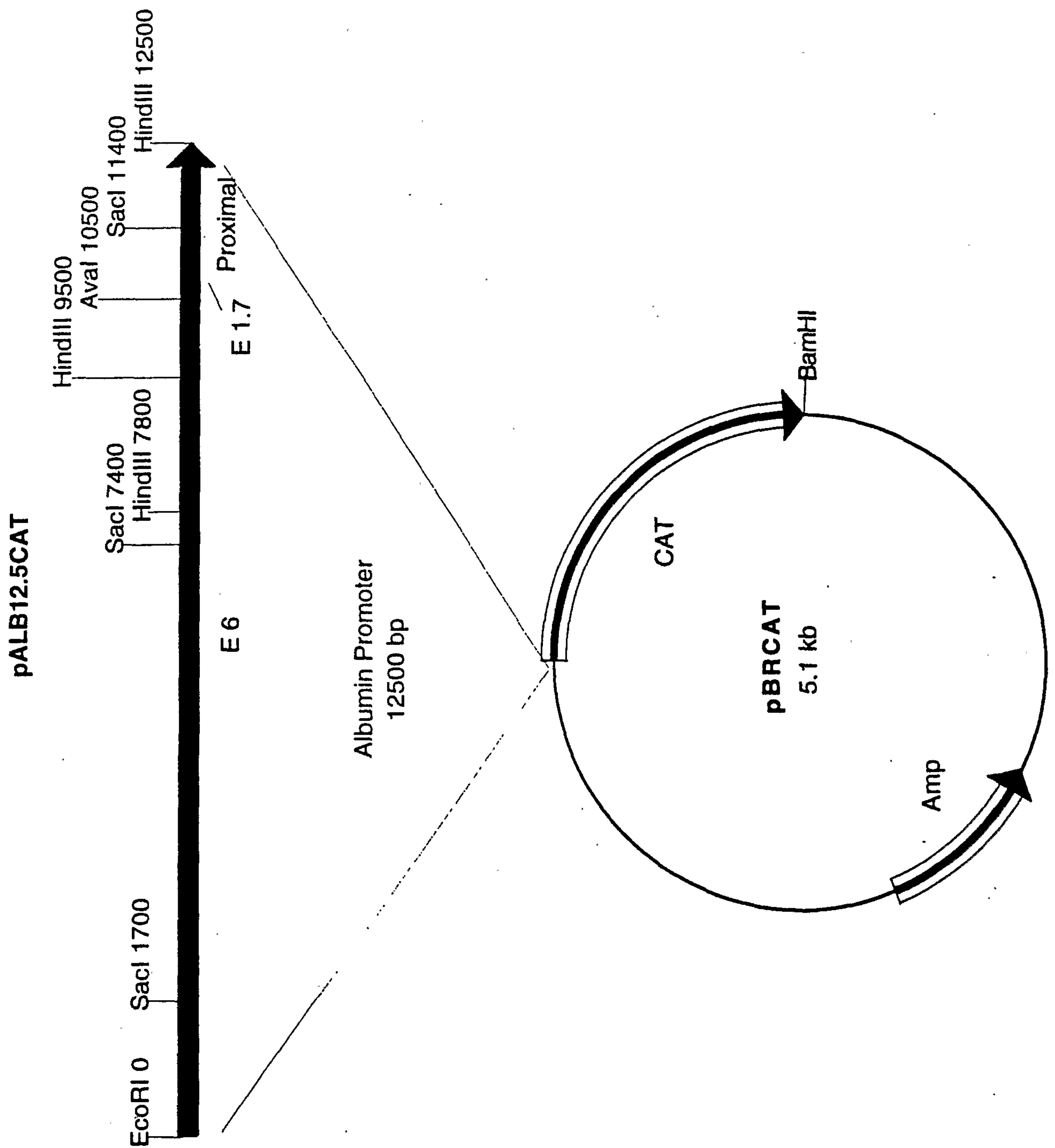


FIG. 37

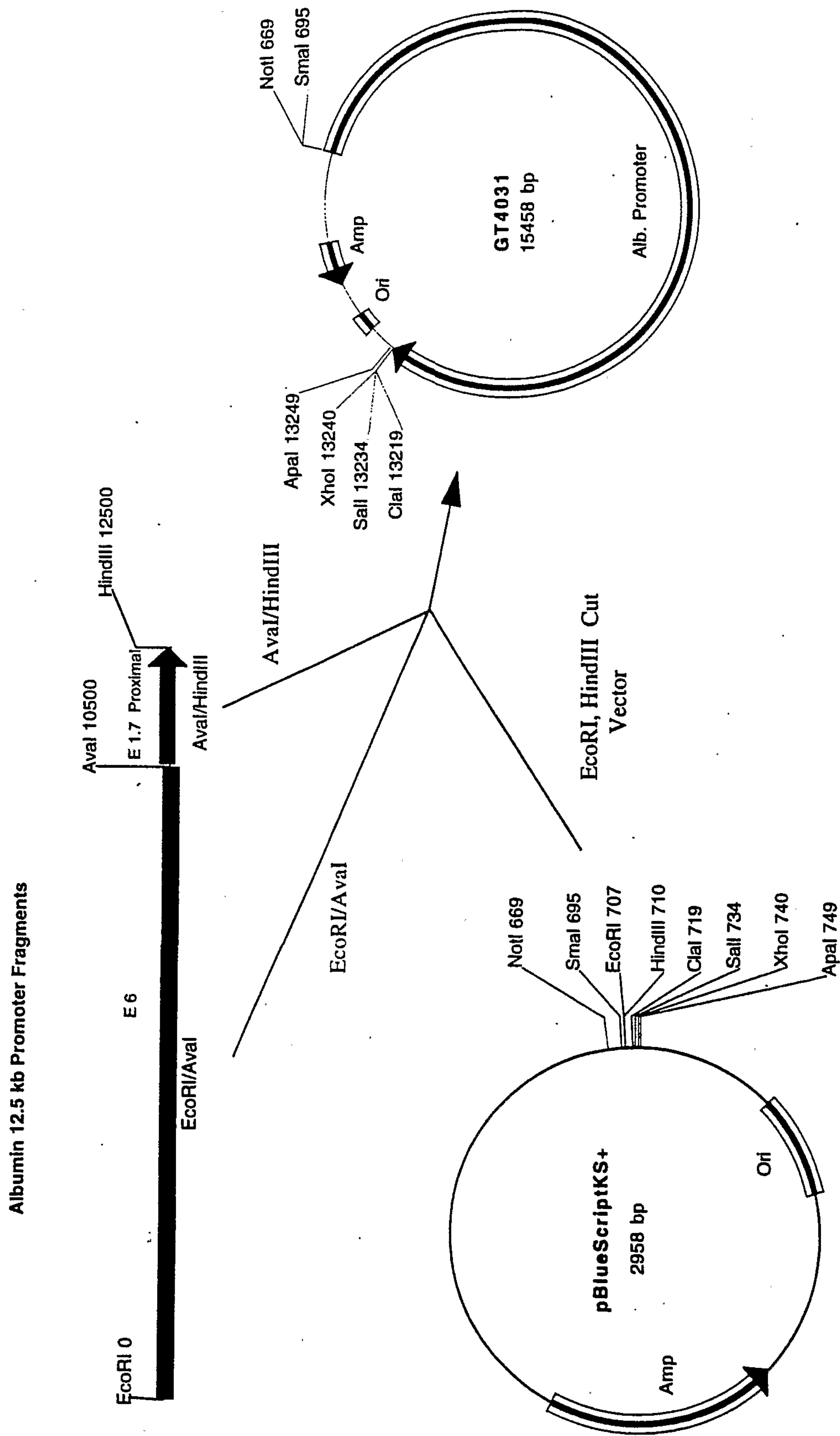


FIG. 38

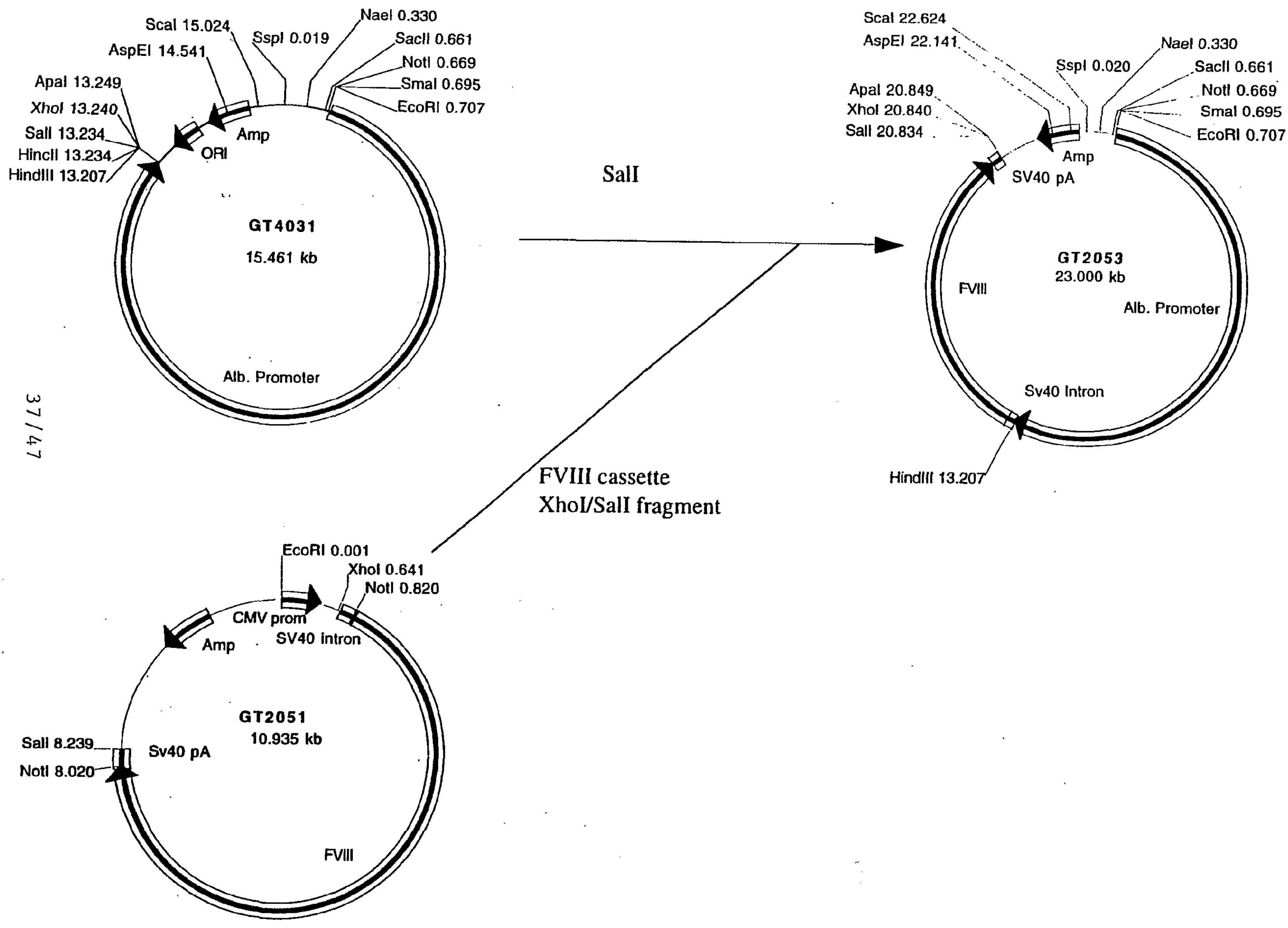
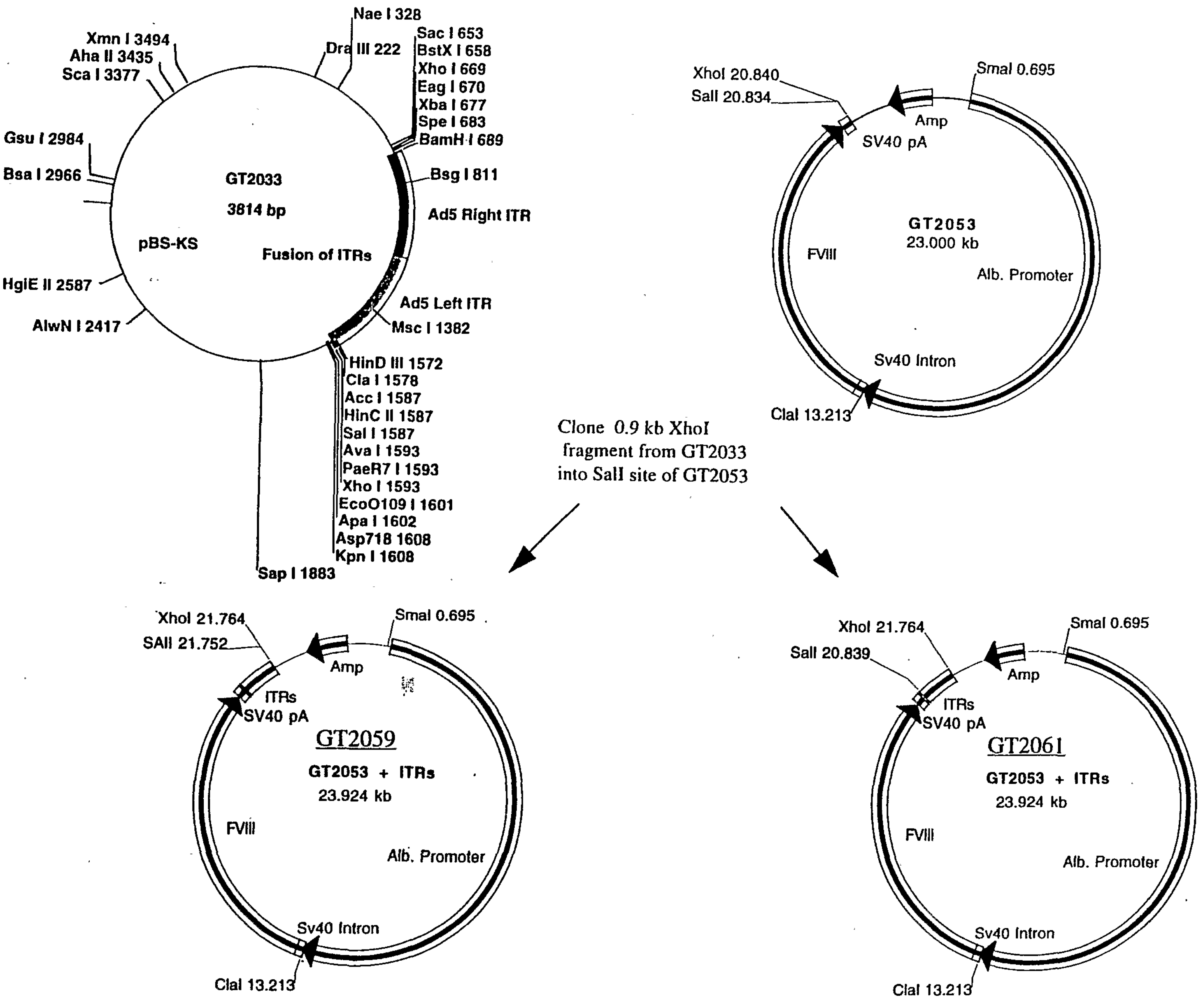


FIG. 39



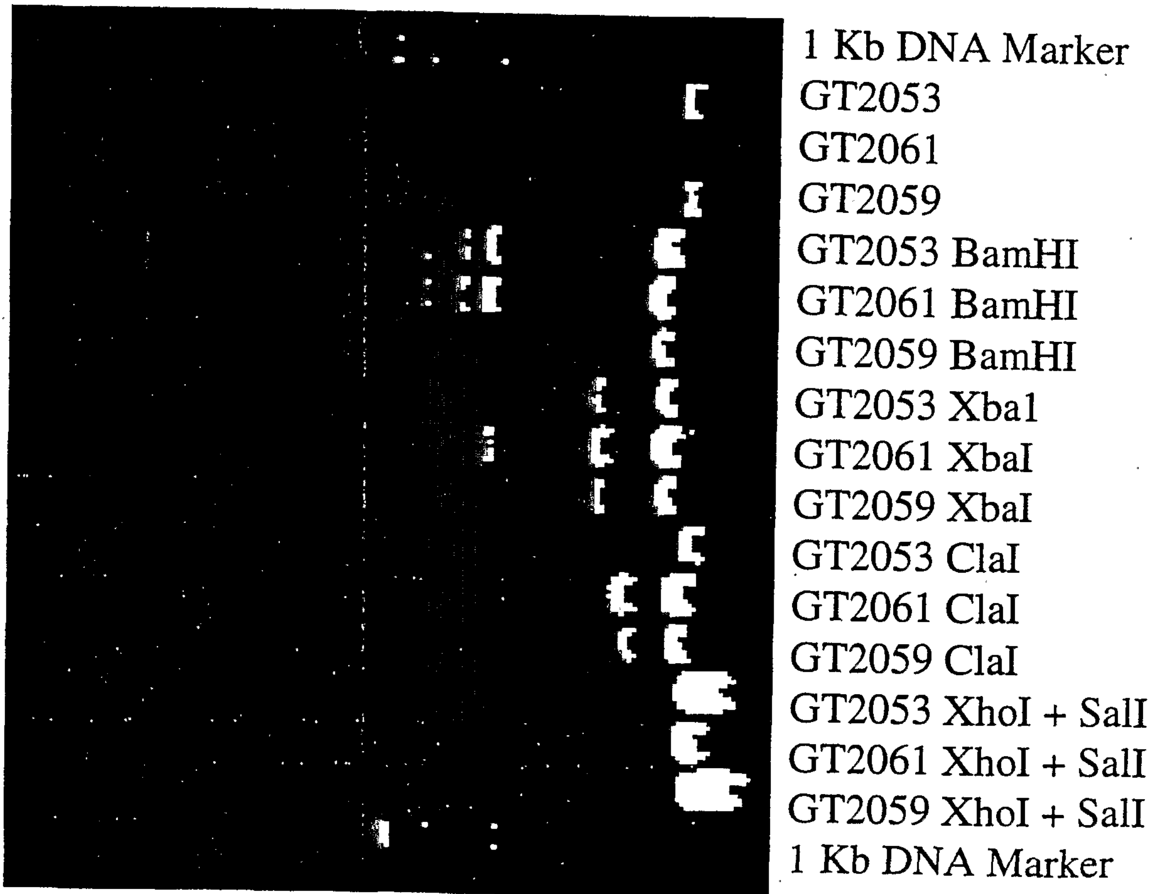


Fig 40.

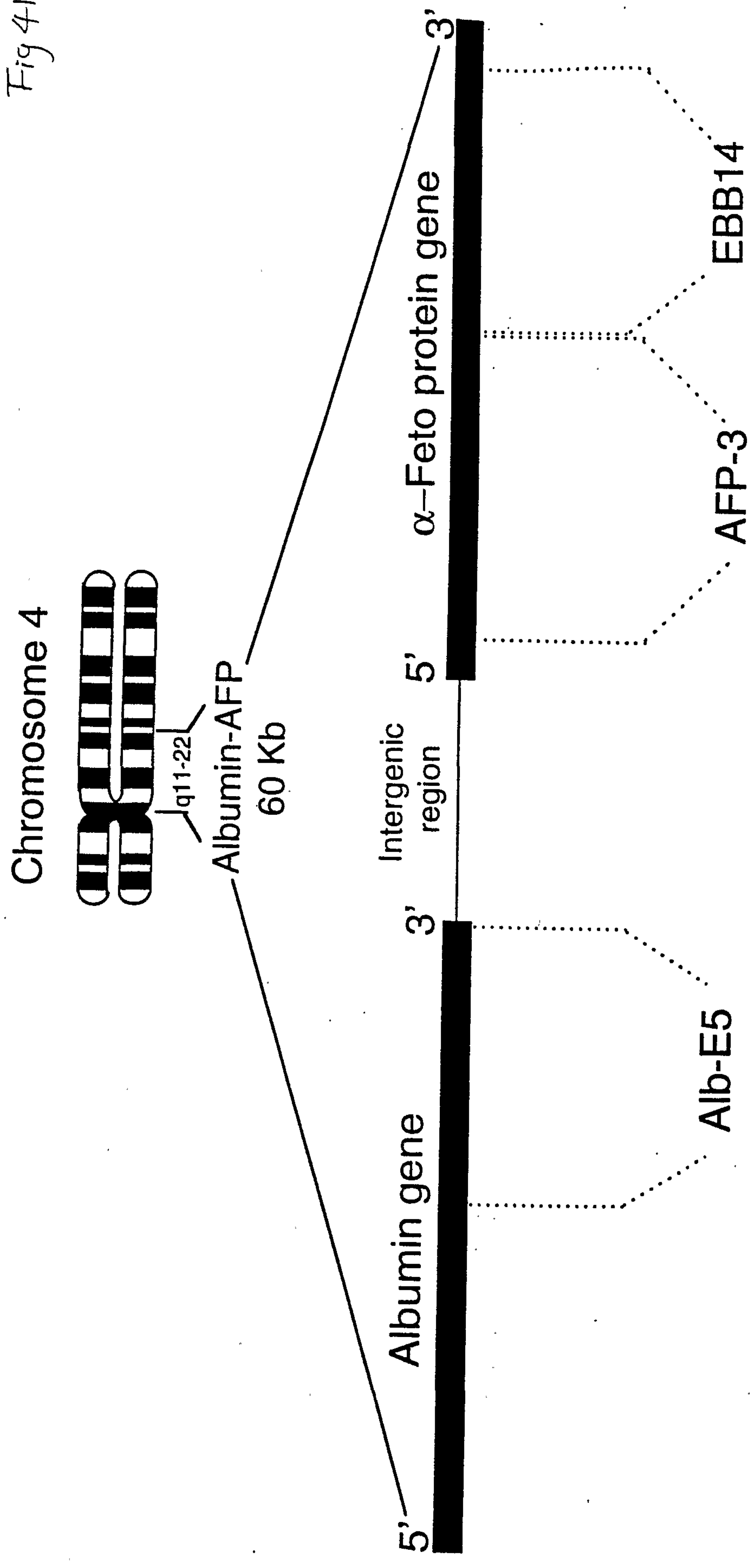


Fig 41.
Chromosome 4
q11-22
Albumin-AFP
60 Kb
3'
α–Feto protein gene
5'
Intergenic region
3'
Albumin gene
5'
EBB14
AFP-3
Alb-E5

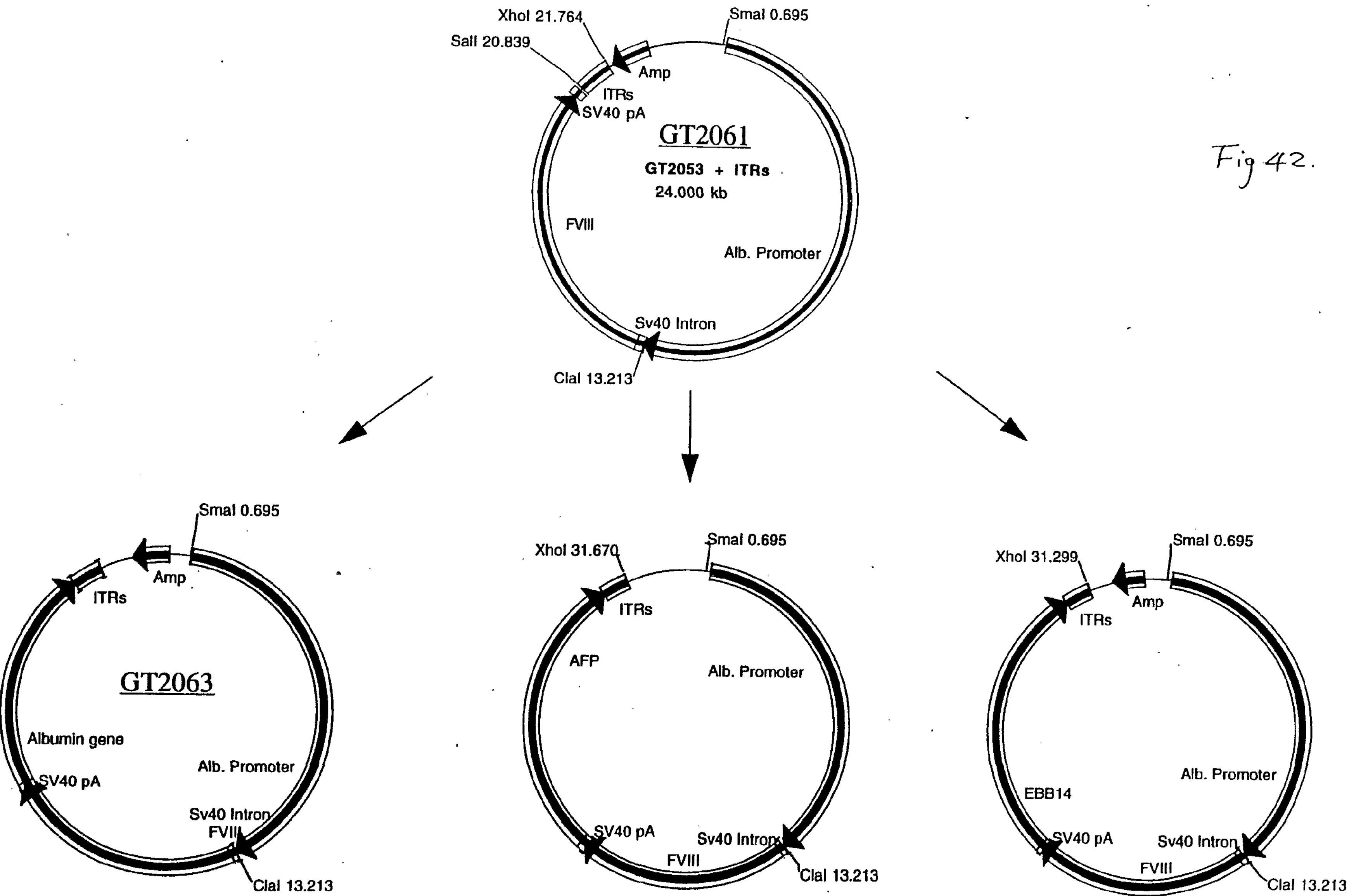


Fig 42.
GT2061
GT2053 + ITRs
24.000 kb
XhoI 21.764
SalI 20.839
SmaI 0.695
Amp
ITRs
SV40 pA
FVIII
Alb. Promoter
Sv40 Intron
ClaI 13.213
GT2063
SmaI 0.695
Amp
ITRs
Albumin gene
SV40 pA
Alb. Promoter
Sv40 Intron
FVIII
ClaI 13.213
XhoI 31.670
SmaI 0.695
ITRs
AFP
Alb. Promoter
SV40 pA
Sv40 Intron
FVIII
ClaI 13.213
XhoI 31.299
SmaI 0.695
Amp
ITRs
EBB14
Alb. Promoter
SV40 pA
Sv40 Intron
FVIII
ClaI 13.213

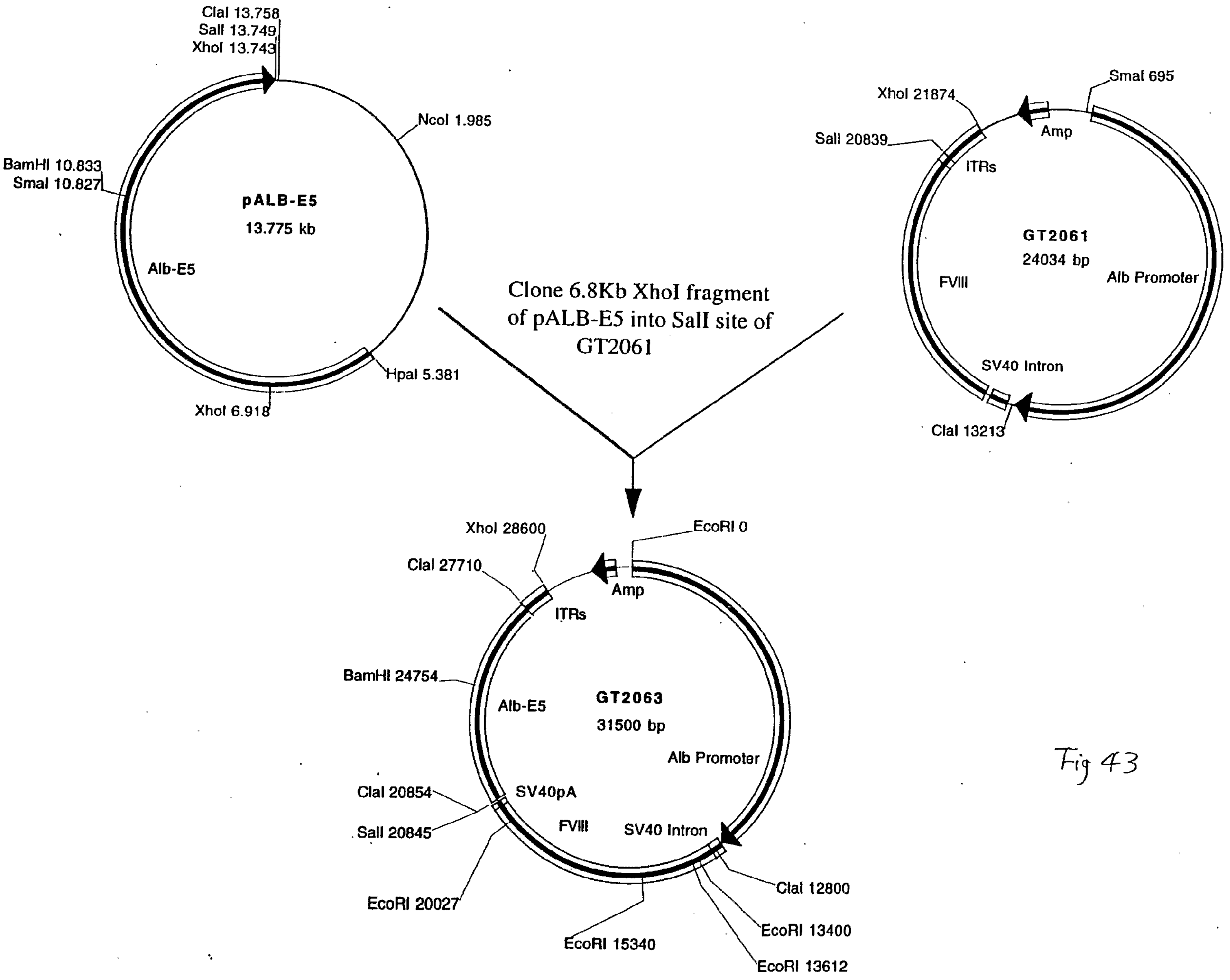
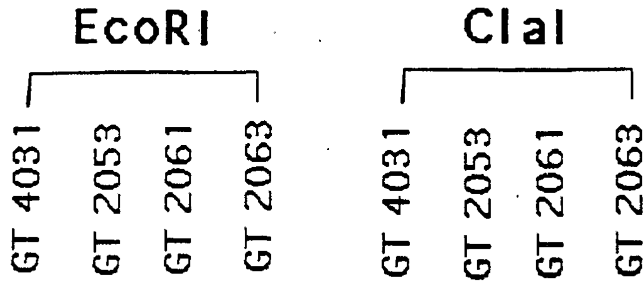


Fig 43

Fig 44.

Mini Ad-FVIII Vector Construction



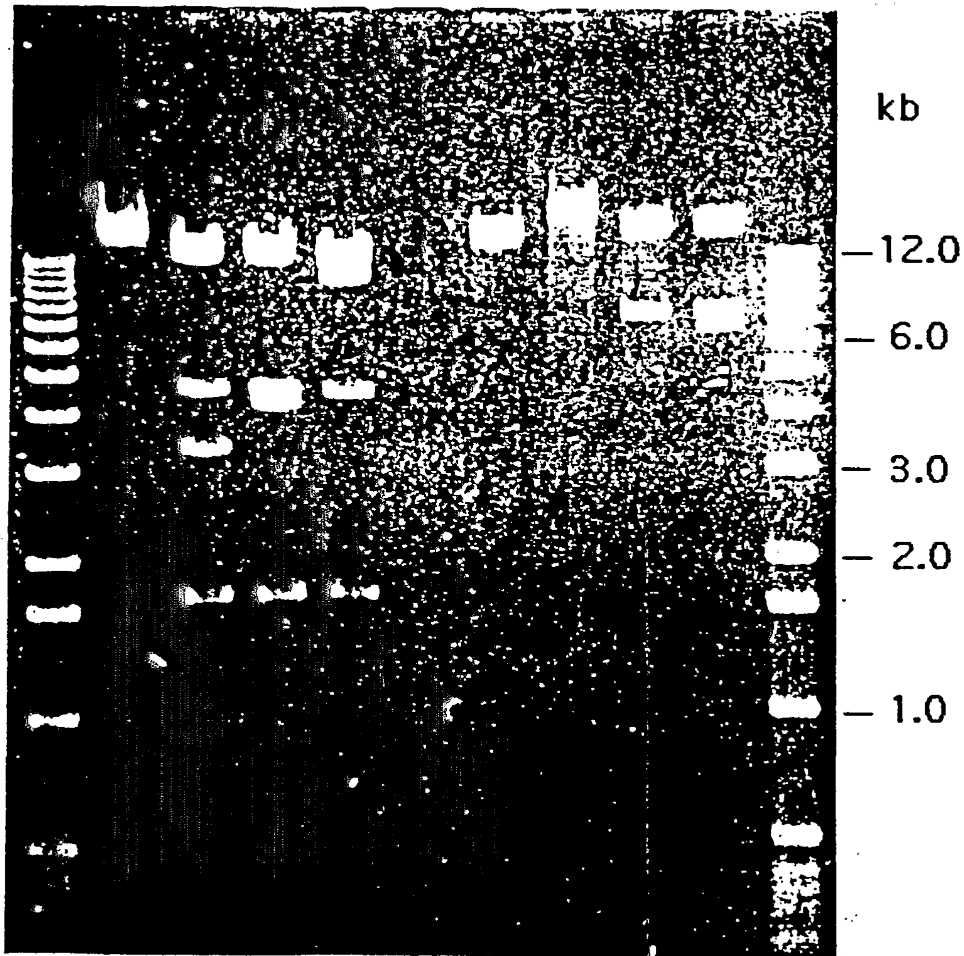


FIG. 45

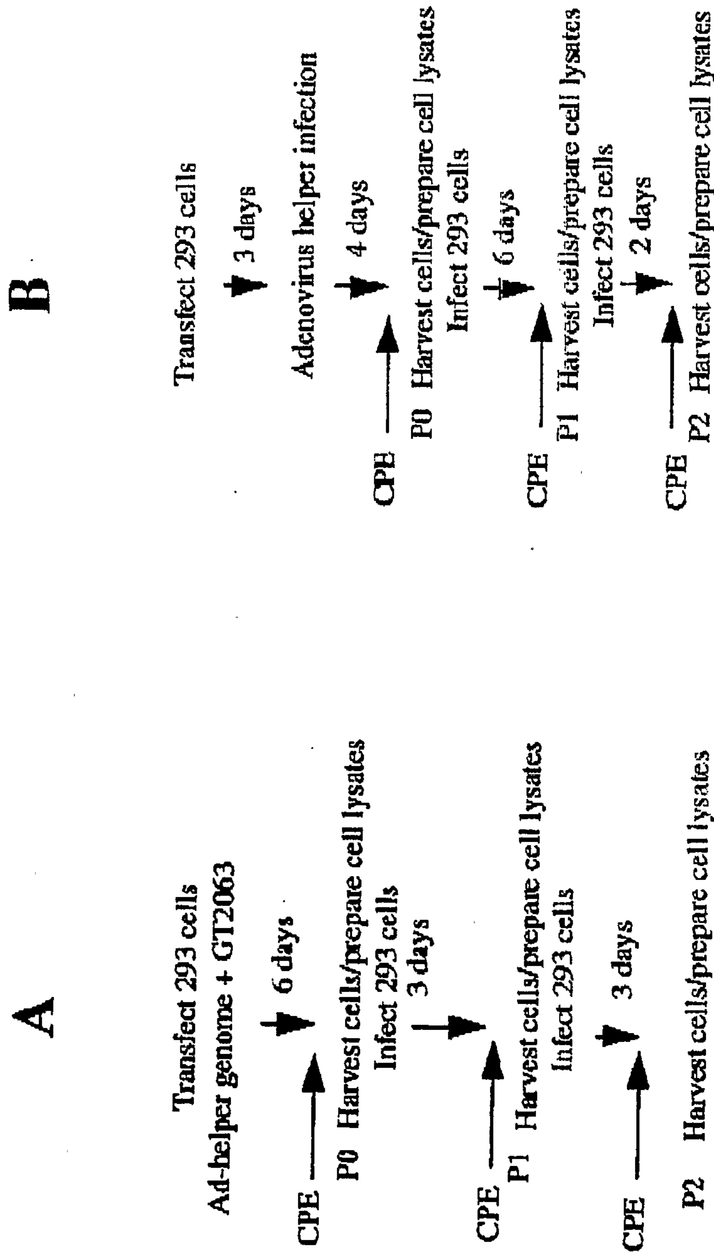


Fig 46.

Marke

1

2

3

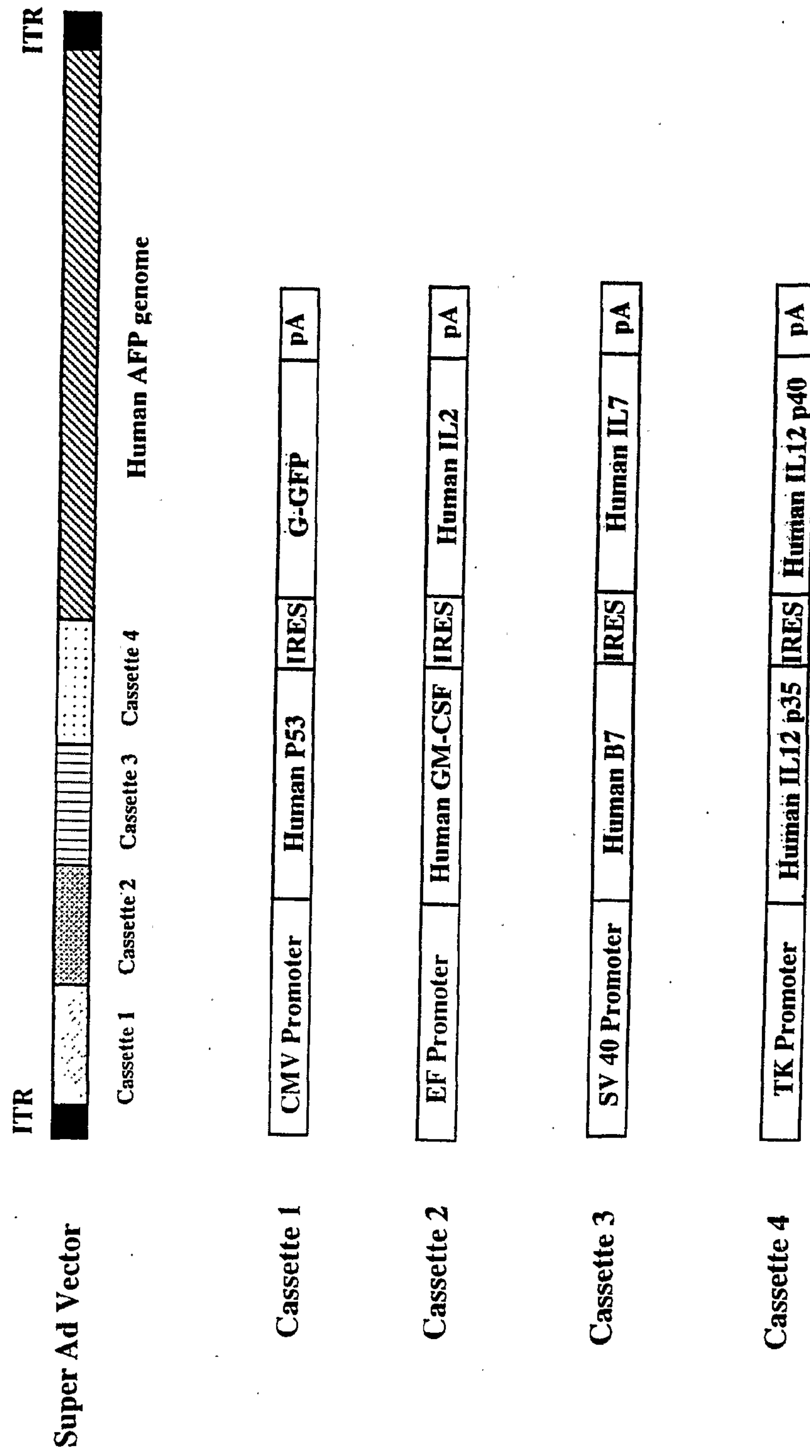
4

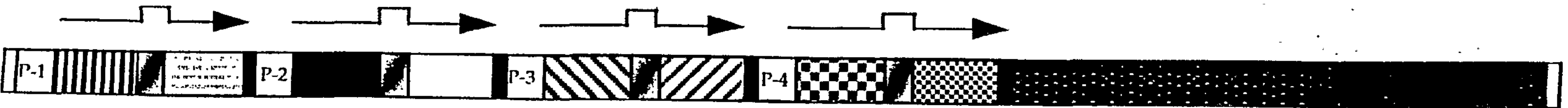
5

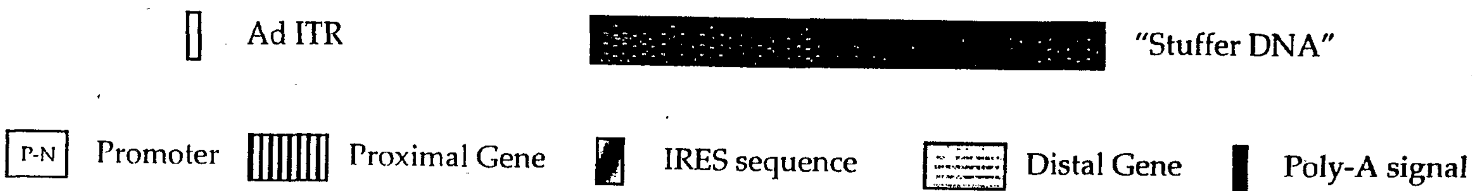
6

Fig 47

The First Generation of Anti-Cancer Super Ad Vector



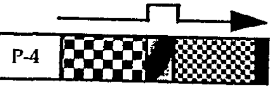

POTENTIAL SECOND GENERATION ADENO-SUPER CANCER VECTOR
P-1
P-2
P-3
P-4


Ad ITR
"Stuffer DNA"
P-N
Promoter
Proximal Gene
IRES sequence
Distal Gene
Poly-A signal

P-1
SV40 e.p.-->B7-1--IRES--IL-7

P-2
CMV e.i.p.-->IL-2--IRES--P53

P-3
TK p.-->ICAM-1--IRES--GM-CSF


P-4
EF1 p.-->anti-TGFβ—IRES--SCA-CTLA-4

Alpha Fetoprotein