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(57) Abrégé/Abstract:
Methods and compositions for producing a mammal capable of expressing an exogenously supplied gene in cells of the airway are disclosed. Lipid carrier-nucleic acid complexes are prepared then delivered via aerosol to the lung airway. The invention provides a direct method for transforming pulmonary cells as a means for treating disorders of the lung as for providing a means for delivering substances systematically following expression in the lung.

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TRANSFECTION OF LUNG VIA AEROSOLIZED TRANSGENE DELIVERY

INTRODUCTION

Technical Field

The present invention relates to methods and compositions for producing a transgenic mammal which comprises an exogenously supplied gene in lung tissue. The gene is supplied by aerosolized delivery, particularly to the airways and alveoli of the lung.

Background

With the advent of molecular cloning techniques, an expanding array of genes with mutations responsible for important human diseases have been identified and isolated. To date, attempts to replace absent or mutated genes in human patients have relied on *ex vivo* techniques. *Ex vivo* techniques include, but are not limited to, transformation of cells in vitro with either naked DNA or DNA encapsulated in liposomes, followed by introduction into a host organ ("*ex vivo*" gene therapy). The criteria for a suitable organ include that the target organ for implantation is the site of the relevant disease, the disease is easily accessible, that it can be manipulated in vitro, that it is susceptible to genetic modification methods and ideally, it should contain either non-replicating cells or cycling stem cells to perpetuate a genetic correction. It also should be possible to reimplant the genetically modified cells into the organism in a functional and stable form. A further requirement for *ex vivo* gene therapy, if for example a retroviral vector is used, is that the cells be pre-mitotic; post-mitotic cells are refractory to infection with retroviral vectors. Exemplary of a target organ which meets the criteria for *in vitro* gene transfer is the mammalian bone marrow.

There are several drawbacks to *ex vivo* therapy. For example, if only differentiated, replicating cells are infected, the newly introduced gene function will be lost as those cells mature and die. *Ex vivo* approaches also can be

used to transfect only a limited number of cells and cannot be used to transfect cells which are not first removed from the body.

Retroviruses, adenoviruses and liposomes have been used in animal model studies in attempts to increase the efficiency of gene transfer; DNA has been introduced into animals by intratracheal (IT), intravenous, intraperitoneal, intramuscular, and intraarterial injection. Expression of introduced genes, either complexed to cationic vectors or packaged in adenoviral vectors has been demonstrated in the lungs of rodents after IT instillation. However, IT injection is invasive and produces a non-uniform distribution of the instilled material; it also is too invasive to be performed repeatedly in humans. It therefore would be of interest to develop a non-invasive delivery technique which also results in deeper penetration of material into the lung than other methods, and can be used to deposit material evenly throughout the airways and alveoli. Such a delivery technique could be used as a means of treatment for genetic disorders, particularly of the lung, via generalized transgene expression in lung cells *in vivo*.

Relevant Literature

Hazinski, *et al.*, *Am. J. Respir. Cell Mol. Biol.* (1991) 4:206-209, relates to liposome-mediated gene transfer of DNA into the intact rodent lung. Three fusion gene constructs were complexed to cationic liposomes including (1) the chloramphenicol acetyltransferase ("CAT") gene linked to a Rous sarcoma virus ("RSV") promoter; (2) the CAT gene linked to a mouse mammary tumor virus ("MMTV") promoter; and (3) a cytomegalovirus-β-galactosidase ("CMV-β-gal") fusion gene. The liposome/DNA complexes were instilled into the cervical trachea of rats and detectable levels of gene expression observed.

Brigham *et al.*, *Am. J. Med. Sci.* (1989) 298:278-281, describes the *in vivo* transfection of murine lungs with the CAT gene using a liposome vehicle. Transfection was accomplished by intravenous, intratracheal or intraperitoneal injection. Both intravenous and intratracheal administration resulted in the expression of the CAT gene in the lungs. However, intraperitoneal administration did not. See, also *Werthers, Clinical Research* (1991) 39:(Abstract).

Canonico *et al.*, *Clin. Res.* (1991) 39:219A describes the expression of the human α-1 antitrypsin gene, driven by the CMV promoter, in cultured bovine lung epithelial cells. The gene was added to cells in culture using cationic liposomes. The experimenters also detected the presence of α-1 antitrypsin in histological sections of the lung of New Zealand white rabbits following the intravenous delivery of gene constructs complexed to liposomes. Yoshimura *et al.* disclose expression of the human cystic fibrosis transmembrane conductance regulator gene in mouse lung after intratracheal liposome-DNA gene transfer. Wolff *et al.*, *Science* (1990) 247:1465-1468 relates to direct transfer of the CAT, β-gal and luciferase genes into mouse skeletal muscle *in vivo*. Gene expression was observed in all three cases. Nabel *et al.*, *Science* (1990) 249:1285-1288, pertains to *in vivo* intra-arterial transfection of pigs with liposomes containing a β-gal expression plasmid. Site-specific gene expression was observed in the arterial wall. None of the above cited art, however, practices or teaches the use of aerosol administration to deliver genes directly to the lung. An example of a review article of human gene therapy procedures is: Anderson, *Science* (1992) 256:808-813.

PCT/US90/01515, having International Publication No. WO 90/11092, describes a method for introducing naked DNA into muscle tissue. Debs *et al.* disclose pentamidine uptake in the lung by aerosolization and delivery in liposomes. *Am Rev Respir Dis* (1987) 135: 731-737.

SUMMARY

Methods and compositions are provided for producing a mammal which comprises an exogenously supplied nucleic acid in its lung cells. The method includes the steps of preparing a lipid carrier-nucleic acid mixture suitable for nebulization, nebulizing the mixture, and depositing the resulting nebulized mixture in the lung of a mammalian host of interest in an amount sufficient to transform cells contacted by the deposited nebulized mixture. The exogenously supplied nucleic acid generally is provided in an expression cassette and includes a coding sequence operably joined to transcriptional and translational regulatory sequences functional in the mammal. The methods and compositions find use particularly for in vivo gene therapy of pulmonary disorders.

In a preferred aspect, the invention provides a composition comprising: a nebulized transfection agent, prepared by nebulizing a mixture comprising complexes between transcription cassettes or expression cassettes and cationic lipid carriers in a pharmaceutically acceptable carrier or diluent, wherein said mixture is substantially free of macroaggregates of said complexes and said lipid carriers have a diameter from 0.1 microns to 10 microns and the ratio of DNA to lipid carrier is in a ratio of 4:1 to 1:10 micrograms DNA to nanomoles cationic lipid and wherein said transcription cassettes or expression cassettes comprise a DNA sequence capable of producing a transcription product in a mammalian cell transfected by said nebulized transfection agent.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 demonstrates that aerosol administration

of pRSV-CAT-DOTMA: cholesterol complexes resulted in expression of the CAT gene in mouse lungs. Lanes 1-3 were derived from mice receiving no treatment; lanes 4-6 represent mice administered 0.5 mg pRSV-CAT with 1.0 μmole DOTMA-cholesterol liposomes; lanes 7-9 were derived from mice receiving 2.0 mg pRSV-CAT alone; and lanes 10-12 represent mice given 2.0 mg pRSV-CAT with 4.0 μmol DOTMA-cholesterol liposomes in a 2 to 1 molar ratio. The CAT gene is not normally present in mammalian cells; lanes 10-12 show spots indicative of CAT activity (the positive spots in lanes 10-11 are faint and do not reproduce well in the figure). The results thus indicate that the lung was successfully transfected by the pRSV-CAT DOTMA-cholesterol:liposome aerosol. The results also show that neither aerosol administration of the pRSV-CAT alone, nor a lower aerosol dose of pRSV-CAT: DOTMA-cholesterol complexes produce detectable expression of the CAT gene in mouse lungs. Thus, both the cationic liposome carrier, and a sufficient dose of DOTMA:liposome-RSV-CAT DNA complexes are required to produce transgene expression in the lung after aerosol administration. Maximum transgene expression is achieved by complexing the liposomes and DNA together at an appropriate ratio and in an appropriate diluent.

Figure 2 shows the results of an experiment where mice were administered 12 mg of pCIS-CAT complexed to 24 μmoles of DOTMA/DOPE 1:1 liposomes. Lanes 1-3 show the results from animals administered the aerosol in an Intox-designed nose-only aerosol exposure chamber; lanes 4-7 are derived from mice exposed to the aerosol in a modified mouse cage; and lanes 8-10 show the results from animals placed in a smaller modified cage after being put in restrainers originally constructed for use in the Intox chamber.

Figure 3 shows construction of pZN13.

Figure 4 shows construction of pZN29.

Figure 5 shows construction of pZN32.

Figure 6 shows the results of immunostaining for intracellular CAT protein in lung sections from mice sacrificed 72 hours after receiving an aerosol containing 12 mg of pCIS-CAT plasmid complexed to 24 μmols of DOTMA:DOPE liposomes (A,B,C,D), or from untreated mice (E,F). The section shown in d was treated with normal rabbit serum in place of anti-CAT antibody. Magnification: A,D (x 50); B,C,E (x 250).

Figure 7 shows CAT activity in lung extracts from mice sacrificed 72 hours after receiving an aerosol containing either 12 mg of CMV-CAT plasmid alone or 12 mg of CMV-CAT plasmid complex to 24 μmols of DOTMA:DOPE (1:1) liposomes. Untreated mice were also assayed.

Figure 8 shows (A) CAT activity in lung extracts from mice sacrificed from one to twenty-one days after receiving an aerosol containing 12 mg of pCIS-CAT plasmid complexed to 24 μmols of DOTMA:DOPE liposomes; and (B) shows CAT activity in several different tissue extracts from mice and indicates that expression of the transgene is lung-specific after aerosolization of DNA-liposome complexes into normal mice sacrificed at the three day time point in Fig. 8A. Control extract contains purified CAT enzyme.

Figure 9 shows Southern blot hybridization of genomic DNA from the lungs of mice sacrificed immediately after receiving an aerosol containing 12 mg of pCIS-CAT plasmid complexed to 24 μmols of DOTMA:DOPE liposomes (lanes 1-4, 6-9) and from an untreated control mouse (lane 5). Samples were digested with the restriction enzyme *Hind*III and probed with a 1.6 kb CAT

fragment (upper panel). The same membrane was hybridized with a 1.1 kb BSU 36-1 single copy probe from a mouse factor VIII.A genomic clone (lower panel).

Figure 10 shows photomicrographs of frozen sections from lungs of control mice (Figures 10B and 10D) and mice treated with pZN32 complexed to DDAB:cholesterol (1:1) liposomes (Figures 10A, 10C, and 10E).

Figure 11 shows a full restriction map for HCMV (Towne) of the immediate early enhancer and promoter region of HCMV (Towne) in Figure 11A and HCMV(AD169) in Figure 11C. Figure 11B shows a sequence comparison of the 2 HCMV promoters. The position of the NcoI site is indicated by an asterisk.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, nucleic acid constructs together with methods of preparation and use are provided which allow for *in vivo* modulation of phenotype and/or genotype of cells in the respiratory tract of a mammalian host following delivery of a sufficient dose of a lipid carrier-nucleic acid aerosol to the host mammal to provide for transfection of host lung cells. The lipid carrier-nucleic acid aerosol is obtained by nebulization of a lipid carrier-nucleic acid sample mixture prepared in a biologically compatible fluid that minimizes aggregation of the lipid carrier-nucleic acid complexes. The methods and compositions can be used to produce a mammal comprising an exogenously supplied gene in lung tissue, particularly alveolar and airway passage cells.

Central to the present invention is the discovery that genes can be delivered to the lung via aerosol administration, and subsequently expressed *in vivo*. The instant invention takes advantage of the use of lipid carriers as a delivery mechanism. Lipid carriers are able to stably bind through charge interactions or entrap and retain nucleic acid and permit a system amenable to nebulization, whereby intact genes can be delivered to specific pulmonary tissues. Lipid carriers include but are not limited to liposome and micelles, as well as biodegradable cationic compounds comprising modified phosphoglycerides particularly alkylphosphoglycerides. Particular sites in the lung are targeted by varying the size of the aerosol particles administered, as discussed more fully below. Targeting agents, such as antibodies directed against surface antigens

expressed on specific pulmonary cell types, can also be covalently conjugated to the lipid carrier surface so that nucleic acid can be delivered to specific cell types. Lipid carriers also allow for the delivery of relatively large amounts of nucleic acid, without a toxic effect, such that therapeutically effective amounts of the desired protein can be expressed *in vivo*. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger *et al.*, in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

Lipid carriers, particularly liposomes, have been used effectively, particularly to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcription factors and other cellular effectors into a variety of cultured cell lines and animals. In addition, successful clinical trials examining the effectiveness of liposome-mediated delivery of small drug molecules and peptides which act extracellularly have been reported. However, while the basic methodology for using liposome-mediated vectors is well developed and has been shown to be safe, the technique previously has not been developed for aerosolized delivery of nucleic acid to pulmonary tissue for *in vivo* gene therapy. By *in vivo* gene therapy is meant transcription and/or translation of exogenously supplied nucleic acid sequences to prevent, palliate and/or cure animal or human disease.

In addition to the discovery that transformation of lung cells can be obtained using aerosolized lipid carrier-nucleic acid complexes, several factors have been identified that can affect the relative ability of particular lipid carrier-nucleic acid complexes to provide transformation of lung cells following aerosolized delivery of a solution containing the lipid carrier-nucleic acid constructs and to achieve a high level of expression where that is the desired endpoint. These factors include (1) preparation of a solution that prior to or during nebulization will not form macroaggregates and wherein the nucleic acid is not sheared into fragments and (2) preparation both of lipid carriers and of expression constructs that provide for predictable transformation of host lung cells following aerosolization of the lipid carrier-nucleic acid complex and administration to the host animal. Other factors include the lipid carrier:nucleic

acid ratio in the solution for nebulization and the diluent used to prepare the solution. These factors are discussed in detail below.

Aerosol delivery of nucleic acid-lipid carrier complexes provides a number of advantages over other modes of administration. For example, aerosol administration can serve to reduce host toxicity. Such an effect has been observed with the delivery of substances such as pentamidine and cytokines, which can be highly toxic when delivered systematically, but are well tolerated when aerosolized. Additionally, the results in rodents with aerosolized pentamidine accurately predicted results in human patients with AIDS treated with aerosolized pentamidine. See, for example, Debs *et al.*, *Antimicrob. Agents Chemother.* (1987) 31:37-41; Debs *et al.*, *Amer. Rev. Respir. Dis.* (1987) 135:731-737; Debs *et al.*, *J. Immunol.* (1988) 140:3482-3488; Montgomery *et al.*, *Lancet* (1987) 11:480-483; Montgomery *et al.*, *Chest* (1989) 95:747-751; Leoung *et al.*, *N. Eng. J. Med.* (1990) 323:769-775. Additionally, rapid clearance of circulating liposomes by the liver and spleen reticuloendothelial system is avoided, thereby allowing the sustained presence of the administered substance at the site of interest, the lung. Serum induced inactivation of the therapeutic agent is also reduced. This method of transfecting lung cells also avoids exposure of the host mammal's gonads, thus avoiding transfection of germ line cells.

Other advantages of the subject invention include ease of administration i.e., the host mammal simply inhales the aerosolized lipid carrier-nucleic acid solution into the intended tissue, the lung. Further, by varying the size of the nebulized particles some control may also be exercised over where in the lung the aerosol is delivered. Delivery may be extended over a long time period. Thus, there is a significant increase in the time period that target cells are exposed to the expression constructs. Distribution of the aerosol is even throughout areas of the lung accessible to the spray. These advantages are significant, particularly when compared to other routes of administration such as intratracheal delivery which is invasive, the expression constructs are delivered in a bolus which may disrupt the mucous barrier and additionally may result in pooling of the introduced fluid in areas of the lung at lower elevation. Further,

damage from insertion of the intratracheal tube may alter the ability of cells coming into contact with the expression constructs to be transfected.

The type of vector used in the subject application may also be an advantage. For example, most gene therapy strategies have relied on transgene insertion into retroviral or DNA virus vectors. Potential disadvantages of retrovirus vectors, as compared to the use of lipid carriers, include the limited ability of retroviruses to mediate *in vivo* (as opposed to *ex vivo*) transgene expression; the inability of retrovirus vectors to transfect non-dividing cells; possible recombination events in replication-defective retrovirus vectors, resulting in infectious retroviruses; possible activation of oncogenes or inhibition of tumor suppressor genes due to the random insertion of the transgene into host cell genomic DNA; size limitations (less than 15 kb of DNA can be packaged in a retrovirus vector, whereas lipid carriers can be used to deliver sequences of DNA of ≥ 250 kb to mammalian cells) and potential immunogenicity of the viral vectors leading to a host immune response against the vector. In addition, all *ex vivo* approaches require that the cells removed from the body be maintained in culture for a period of time. While in culture, cells may undergo deleterious or potentially dangerous phenotypic and/or genotypic changes. Adenovirus and other DNA viral vectors share several of the above potential limitations. Particularly for human use, but also for repeated veterinary use, biodegradable lipid carriers may be used which are metabolized by the host mammal to naturally occurring compounds that are non-toxic to the host and/or are readily excreted.

The nucleic acid constructs generally will be provided as expression cassettes which will include as operably linked components in the direction of transcription, a transcriptional initiation region, a nucleic acid sequence of interest and a transcriptional termination region wherein the transcriptional regulatory regions are functional in the mammalian host lung cell. An intron optionally may be included in the construct, preferably ≥ 100 bp and placed 5' to the coding sequence. Generally it is preferred that the construct not become integrated into the host cell genome and it is introduced into the host as part of a non-integrating expression cassette. A coding sequence is "operably linked to" or "under the control of" transcriptional and/or translational regulatory regions in a cell when

DNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, either a sense strand or an antisense strand. Thus, the nucleic acid sequence includes DNA sequences which encode polypeptides which are directly or indirectly responsible for a therapeutic effect, as well as genes coding for active nucleotide sequences such as antisense sequences and ribozymes.

The constructs for use in the invention include several forms, depending upon the intended use of the construct. Thus, the constructs include vectors, transcriptional cassettes, expression cassettes and plasmids. The transcriptional and translational initiation region (also sometimes referred to as a "promoter,"), preferably comprises a transcriptional initiation regulatory region and a translational initiation regulatory region of untranslated 5′ sequences, "ribosome binding sites," responsible for binding mRNA to ribosomes and translational initiation. It is preferred that all of the transcriptional and translational functional elements of the initiation control region are derived from or obtainable from the same gene. In some embodiments, the promoter will be modified by the addition of sequences, such as enhancers, or deletions of nonessential and/or undesired sequences. By "obtainable" is intended a promoter having a DNA sequence sufficiently similar to that of a native promoter to provide for the desired specificity of transcription of a DNA sequence of interest. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences.

For the transcriptional initiation region, or promoter element, any region may be used with the proviso that it provides the desired level of transcription of the DNA sequence of interest. The transcriptional initiation region may be native to or homologous to the host cell, and/or to the DNA sequence to be transcribed, or foreign or heterologous to the host cell and/or the DNA sequence to be transcribed. By foreign to the host cell is intended that the transcriptional initiation region is not found in the host into which the construct comprising the transcriptional initiation region is to be inserted. By foreign to the DNA sequence is intended a transcriptional initiation region that is not normally associated with the DNA sequence of interest. Efficient promoter elements for transcription initiation include the SV40 (simian virus 40) early promoter, the RSV (Rous

sarcoma virus) promoter, the Adenovirus major late promoter, and the human CMV (cytomegalovirus) immediate early 1 promoter.

Inducible promoters also find use with the subject invention where it is desired to control the timing of transcription. Examples of promoters include those obtained from a β-interferon gene, a heat shock gene, a metallothionein gene or those obtained from steroid hormone-responsive genes, including insect genes such as that encoding the ecdysone receptor. Such inducible promoters can be used to regulate transcription of the transgene by the use of external stimuli such as interferon or glucocorticoids. Since the arrangement of eukaryotic promoter elements is highly flexible, combinations of constitutive and inducible elements also can be used. Tandem arrays of two or more inducible promoter elements may increase the level of induction above baseline levels of transcription which can be achieved when compared to the level of induction above baseline which can be achieved with a single inducible element.

Generally, the regulatory sequence comprises DNA up to about 1.5 Kb 5′ of the transcriptional start of a gene, but can be significantly smaller. This regulatory sequence may be modified at the position corresponding to the first codon of the desired protein by site-directed mutagenesis (Kunkel TA, 1985, *Proc. Natl. Acad. Sci. (USA)*, 82:488-492) or by introduction of a convenient linker oligonucleotide by ligation, if a suitable restriction site is found near the N-terminal codon. In the ideal embodiment, a coding sequence with a compatible restriction site may be ligated at the position corresponding to codon #1 of the gene. This substitution may be inserted in such a way that it completely replaces the native coding sequence and thus the substituted sequence is flanked at its 3′ end by the gene terminator and polyadenylation signal.

Transcriptional enhancer elements optionally may be included in the expression cassette. By transcriptional enhancer elements is intended DNA sequences which are primary regulators of transcriptional activity and which can act to increase transcription from a promoter element, and generally do not have to be in the 5′ orientation with respect to the promoter in order to enhance transcriptional activity. The combination of promoter and enhancer element(s) used in a particular expression cassette can be selected by one skilled in the art to

maximize specific effects. Different enhancer elements can be used to produce a desired level of transgene expression in a wide variety of tissue and cell types. For example, the human CMV immediate early promoter-enhancer element can be used to produce high level transgene expression in many different tissues *in vivo*.

Examples of other enhancer elements which confer a high level of transcription on linked genes in a number of different cell types from many species include enhancers from SV40 and RSV-LTR. The SV40 and RSV-LTR are essentially constitutive. They may be combined with other enhancers which have specific effects, or the specific enhancers may be used alone. Thus, where specific control of transcription is desired, efficient enhancer elements that are active only in a tissue-, developmental-, or cell-specific fashion include immunoglobulin, interleukin-2 (IL-2) and ß-globin enhancers are of interest. Tissue-, developmental-, or cell-specific enhancers can be used to obtain transgene expression in particular cell types, such as B-lymphocytes and T-lymphocytes, as well as myeloid, or erythroid progenitor cells. Alternatively, a tissue-specific promoter such as that derived from the human cystic fibrosis transmembrane conductance regulator (CFTR) gene can be fused to a very active, heterologous enhancer element, such as the SV40 enhancer, in order to confer both a high level of transcription and tissue-specific transgene transcription. In addition, the use of tissue-specific promoters, such as LCK, may allow targeting of transgene transcription to T lymphocytes. Tissue specific transcription of the transgene may be important, particularly in cases where the results of transcription of the transgene in tissues other than the target tissue would be deleterious.

Tandem repeats of two or more enhancer elements or combinations of enhancer elements may significantly increase transgene expression when compared to the use of a single copy of an enhancer element; hence enhancer elements find use in the expression cassette. The use of two different enhancer elements from the same or different sources flanking or within a single promoter can in some cases produce transgene expression in each tissue in which each individual enhancer acting alone would have an effect, thereby increasing the number of tissues in which transcription is obtained. In other cases, the presence of two different enhancer elements results in silencing of the enhancer effects. Evaluation

of particular combinations of enhancer elements for a particular desired effect or tissue of expression is within the level of skill in the art. Although generally it is not necessary to include an intron in the expression cassette, an intron comprising a 5′ splice site (donor site) and a 3′ splice site (acceptor site) separated by a sufficient intervening sequence to produce high level, extended *in vivo* expression of a transgene administered iv or ip can optionally be included. Generally, an intervening sequence of about 100bp produces the desired expression pattern and/or level, but the size of the sequence can be varied as needed to achieve a desired result. The optional intron placed 5' to the coding sequence results in high level extended *in vivo* expression of a transgene administered iv or ip but generally is not necessary to obtain expression. Optimally, the 5' intron specifically lacks cryptic splice sites which result in aberrantly spliced mRNA sequences. If used, the intron splice donor and splice acceptor sites, arranged from 5′ to 3′ respectively, are placed between the transcription initiation site and the translational start codon as diagrammed in (1), below.

Consensus sequences for the 5′ and 3′ splice sites used in RNA splicing

5′ exon		intron		3′ exon[1]	
C	A	U U U U U U U U U U U C		G	(1)
5′—or A G GU or A G U —		— or or or or or or or or or or or N or AG		or—3′	
A	G	C C C C C C C C C C C U		A	
consensus sequence for 5′ splice site ("donor site")		consensus sequence for 3′ splice site ("acceptor site")			

[1] The sequence given is that for the RNA chain; the nearly invariant GU and AG dinucleotides at either end of the intron are shaded.

Alternatively, the intervening sequence may be placed 3′ to the translational stop codon and the transcriptional terminator or inside the coding region. The intron can be a hybrid intron with an intervening sequence or an intron taken from a genomic coding sequence. An intron 3' to the coding region, a 5' intron which is of less than 100 bp, or an intron which contains cryptic splice sites may under certain condition substantially reduce the level of transgene expression produced *in vivo*. However, unexpectedly, a high level of *in vivo*

expression of a transgene can be achieved using a vector that lacks an intron. Such vectors therefore are of particular interest for *in vivo* transfection.

In some cases, it may be desirable to use constructs that produce long term transgene expression *in vivo*, either by integration into host cell genomic DNA at high levels or by persistence of the transgene in the nucleus of cells *in vivo* in stable, episomal form. Integration of the transgene into genomic DNA of host cells *in vivo* may be facilitated by administering the transgene in a linearized form (either the coding region alone, or the coding region together with 5' and 3' regulatory sequences, but without any plasmid sequences present). Additionally, in some instances, it may be desirable to delete or inactivate a mutant gene and replace it with a desired transgene. This may be achieved by using an expression cassette suitable for homologous recombination *in vivo*. Thus, for example, a linearized plasmid comprising an expression cassette may be used such as is described in European patent applications 88/201743.7 and EP89/202106.4. These applications disclose a plasmid for targeting of a specific gene. For the present application, a linear plasmid can be constructed wherein the replacement gene is flanked by 5' and 3' sequences which are sufficiently homologous with the 5' and 3' sequences of the defective gene to provide for homologous recombination. Where it desired to insert the replacement gene in the mutant gene (thereby inactivating it) a means for selection is included within the 5' and 3' flanking sequences of the plasmid.

The incidence of transgene integration into genomic DNA may be increased by incorporating a purified retroviral enzyme, such as the HIV-1 integrase enzyme, into the lipid carrier-DNA complex. Appropriate flanking sequences are placed at the 5' and 3' ends of the transgene DNA. These flanking sequences have been shown to mediate integration of the HIV-1 DNA into host cell genomic DNA in the presence of HIV-1 integrase. Alternatively, the duration of the transgene expression *in vivo* can be prolonged by the use of constructs that contain non-transforming sequences of a virus such as Epstein-Barr virus, sequences such as oriP and EBNA-1 which appear to be sufficient to allow heterologous DNA to be replicated as an episome in mammalian cells (Buhans *et al.*, Cell (1986) 52:955).

Downstream from and under control of the transcriptional initiation regulatory regions is a multiple cloning site for insertion of a nucleic acid sequence of interest which will provide for one or more alterations of host genotype and modulation of host phenotype. Conveniently, the multiple cloning site may be employed for a variety of nucleic acid sequences in an efficient manner. The nucleic acid sequence inserted in the cloning site may have any open reading frame encoding a polypeptide of interest, for example, an enzyme, with the proviso that where the coding sequence encodes a polypeptide of interest, it should lack cryptic splice sites which can block production of appropriate mRNA molecules and/or produce aberrantly spliced or abnormal mRNA molecules. The nucleic acid sequence may be DNA; it also may be a sequence complementary to a genomic sequence, where the genomic sequence may be one or more of an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, for example splicing, or translation.

A number of nucleic acid sequences are of interest for use in *vivo* gene therapy of lung diseases or diseases of other tissues. When it is desired to have an extra-pulmonary effect, nucleic acid providing for secretory leader sequence is included in the expression cassette. Where the nucleic acid codes for a polypeptide, the polypeptide may be one which is active intracellularly, a transmembrane protein, or it may be a secreted protein. It may also code for a mutant protein for example are which is normally secreted but which has been altered to act intracellularly. The nucleic acid may also be a DNA sequences coding for mRNA (antisense or ribozyme sequences such as those to HIV-REV or BCR-ABL sequences) or for proteins such as transdominant negative mutants which specifically prevent the integration of HIV genes into the host cell genomic DNA, replication of HIV sequences, translation of HIV proteins, processing of HIV mRNA or virus packaging in human cells; the LDL (low density lipoprotein) receptor, which specifically lowers serum cholesterol, and which can reduce the risk of heart attack in individuals with elevated serum cholesterol levels, and proteins such as granulocyte macrophage colony stimulating factor (GM-CSF) which can stimulate the production of white blood cells from the bone marrow of

immunocompromised patients and produce significant anti-tumor activity or cystic fibrosis transmembrane conductance regulator (CFTR) for treatment cystic fibrosis. These, or other beneficial (therapeutic) nucleic acid sequences can be expressed in appropriate cells *in vivo* using this invention.

Examples of beneficial therapeutic nucleic acid sequences are those encoding molecules have superoxide dismutase activity or catalase activity to protect the lung from oxidant injury; endothelial prostaglandin synthase to produce prostacyclin and prostaglandin E2; and antiprotease alpha-1 antitrypsin. Thus, this approach could dramatically improve the treatment of acquired immune deficiency syndrome (AIDS), cystic fibrosis, cancer, heart disease, autoimmune diseases and a variety of life threatening infections. For the treatment AIDS, anti-TAT, REV TAR or other critical anti-HIV sequences may be used, particularly for expression of the appropriate coding sequences in T lymphocytes, macrophages and monocytes which can be achieved following iv administration of the appropriate coding sequences; expression of wild-type CFTR gene in the lungs of cystic fibrosis patients (see Collins, *Science* (1992) 256:774-783) CFTR cDNA can be obtained from Dr. Collins at University of Michigan or Dr. Tsui at Toronto Sick Children's Hospital; expression of wild-type p53 in tumors of cancer patients with absent or aberrant expression of this gene, p53 is obtainable from Dr. Vogelstein at John Hopkins Univ; antisense sequences to over-expressed, transforming oncogenes, such as myc or ras in tumors; genes which block activity of activated T cell clones which attack myelin in multiple sclerosis or other targets in autoimmune diseases. A T-cell lymphocyte clone activated to recognize and attack myelin can be targeted by using an anti-sense sequence, ribozyme sequence or transgene coding for a transdominant negative mutant which specifically blocks surface expression on the T-cell of T-cell receptor components which mediate recognition and/or attack of myelin-sheathed cells.

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region may be native to the intended nucleic acid sequence of interest, or may be derived from another source. Convenient termination regions are available and include the 3′ end of a gene terminator and polyadenylation

signal from the same gene from which the 5′ regulatory region is obtained. Adenylation residues, preferably more than 32 and up to 200 or more as necessary may be included in order to stabilize the mRNA. Alternatively, a terminator and polyadenylation signal from different gene/genes may be employed with similar results. Specific sequences which regulate post-transcriptional mRNA stability may optionally be included. For example, certain polyA sequences (Volloch *et al*. *Cell* (1981) 23:509) and ß-globin mRNA elements can increase mRNA stability, whereas certain AU-rich sequences in mRNA can decrease mRNA stability (Shyu *et al*., Genes and Devel. (1989) 3:60). In addition, AU regions in 3′ non-coding regions may be used to destabilize mRNA if a short half-life mRNA is desirable for the gene of interest.

The construct may additionally include sequences for selection, such as a neomycin resistance gene or a dihydrofolate reductase gene and/or signal sequences to regenerate recombinant proteins that are targeted to different cellular compartments or secreted when the wild type sequence is not. Any of a variety of signal sequences may be used which are well-known to those skilled in the art. These signal sequences may allow generation of new vaccine strategies or produce soluble antagonists directed against specific cell surface receptors such as transformed oncogenes. The sequences for selection may be on a separate plasmid and cotransfected with the plasmid carrying the therapeutic nucleic acid. Where a carrier is used, the selection plasmid may be complexed to a different carrier or to the same carrier as the therapeutic plasmid.

The recombinant coding-sequence flanked at its 5′ end by the promoter and regulatory sequences and at its 3′ end by a terminator and regulatory sequences may be introduced into a suitable cloning plasmid (e.g., pUC18, pSP72) for use in direct DNA uptake in host cells following introduction into the host

Isolation of Genes and Construction of Lipid carriers

Nucleic acid sequences for use in the present invention, can be derived from known sources, for example by isolating the nucleic acid from cells containing the desired gene, using standard techniques. Similarly, the gene sequence can be generated synthetically, using standard modes of polynucleotide synthesis, well known in the art. See, e.g. Edge, M.D., *Nature* (1981) 292:756;

Nambair, *et al.*, *Science* (1984) 223:1299; Jay, Ernest, J Biol Chem (1984) 259:6311. Generally, synthetic oligonucleotides are prepared by either the phosphotriester method as described by Edge *et al.*, *Nature* (supra) and Duckworth *et al.*, *Nucleic Acids Res* (1981)9:1691, or the phosphoramidite method as described by Beaucage, S.L., and Caruthers, M.H., *Tet. Letts.* (1981) 22:1859, and Matteucci, M.D., and Caruthers, M.H., *J. Am. Chem. Soc.* (1981) 103:3185, and can be prepared using commercially available automated oligonucleotide synthesizers. The gene sequence can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for expression in the intended host. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair *et al.*, (1984) *Science* 223:1299; Jay *et al.*, (1984) *J. Biol. Chem.* 259:6311.

A particularly convenient method for obtaining nucleic acid for use in the lipid carrier-nucleic acid preparations, is by recombinant means. Thus, the desired gene can be excised from a plasmid carrying the desired gene, using standard restriction enzymes and procedures. Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1950) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using standard techniques. The Klenow fragment fills in at 5' single-stranded overhangs but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture can be extracted with e.g. phenol/chloroform,

and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art; the selection of an appropriate cloning vector is known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Ligation to other sequences is performed using standard procedures, known in the art. For example, ligations can be accomplished in 30 mM Tris-C1 pH 7.8, 10 mM $MgC1_2$, 10mM DTT, 50 μg/ml BSA, and either 1 mM ATP, 0.01-1.0 (Weiss) units T4 DNA ligase at 16°C (for "sticky end" ligation) or 1mM ATP, 0.5-1.0 (Weiss) units T4 DNA ligase at 20°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 30-100 μg/ml total DNA concentration (5-100 nM total end concentration).

The nucleic sequence is placed under the control of a promoter, ribosome binding site and, optionally, an operator (collectively referred to herein as "control" elements), so that the coding sequence is transcribed into RNA in the host tissue transformed by the lipid carrier-nucleic acid. The coding sequence may or may not contain a signal peptide or leader sequence. A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3' terminus by the transcription start codon (ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Nucleic acid "control sequences" or "regulatory regions" refer collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which

collectively provide for the transcription and translation of a coding sequence in a host cell.

The choice of regulatory elements will depend on the host cell which is to be transformed and the type of nucleic acid preparation used. Thus, if the host cells' endogenous transcription and translation machinery will be used to express a polypeptide of interest, control elements functional in the particular host which provide for expression are used. Several promoters for use in mammalian cells are known in the art and include, but are not limited to, a SV40 (Simian Virus 40) early promoter, a RSV (Rous Sarcoma Virus) promoter, an Adenovirus major late promoter, and a human CMV (Cytomegalovirus) immediate early one promoter. Other promoters which may be used include those derived from mouse mammary tumor virus (MMTV, T7, T3, and the like). Particularly useful in the present invention are the RSV promoter and the CMV promoter, particularly the immediate early promoter from the AD169 strain of CMV.

In addition to the above sequences, it may be desirable to add to the nucleic acid construct regulatory sequences which allow for regulation of the expression of the polypeptide of interest sequences. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Such promoters can be used to regulate expression of the transgene by the use of external stimuli such as interferon or glucocorticoids.

Other types of regulatory elements may also be present in the plasmid, for example, enhancer sequences. Such regulatory elements include those obtainable from β-interferon, heat shock, metallothionein or steroid hormone responsive genes, including insect genes such as the ecdysone receptor gene.

Since the arrangement of eukaryotic promoter elements is highly flexible, combinations of constitutive and inducible elements can be used. Tandem arrays of two or more inducible promoter elements may increase the level of induction above baseline levels of transcription which can be achieved with a single inducible element. By transcription enhancer elements are intended DNA sequences which are primary regulators of transcriptional activity which can act to

increase transcription from a promoter element, and generally do not have to be in the 5' orientation with respect to the promoter in order to enhance transcriptional activity.

The combination of promoter and enhancer elements used in a particular nucleic acid construct can be selected by one skilled in the art to maximize specific effects; different enhancer elements can be used to produce a desired level of transgene expression. For example, a tissue specific promoter such as that derived from the human cystic fibrosis transmembrane conductance regulator (CFTR) gene can be used flanking a very active, heterologous enhancer element, such as the SV40 enhancer, in order to obtain both a high level of expression and expression of the transgene primarily in airway epithelial cells in the lung. Tandem repeats of two or more enhancer elements or combinations of enhancer elements may significantly increase transgene expression when compared to the use of a single copy of an enhancer element. The use of two different enhancer elements from the same or different sources, flanking or within a single promoter may be used. Evaluation of particular combinations of enhancer elements for a particular desired effect or expression level is within the knowledge of one skilled in the art. Promoter-enhancer elements which are least partially derived from CMV Townes and/or AD169 strains are of particular interest for providing a high level of expression of a transgene.

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region may be native to the intended nucleic acid sequence of interest, or may be derived from another source. Convenient termination regions are available and include the 3' end of a gene terminator and polyadenylation signal from the same gene from which the 5' regulatory region is obtained. Adenylation residues, preferably more than 32 and up to 200 or more if necessary may be included in order to stabilize the mRNA. Alternatively, terminator and polydenylation signals from a different gene/genes may be employed with similar results. Specific sequences which regulate post-transcriptional mRNA stability may optionally be included. For example, certain polyA sequences (Volloch *et al.*, Cell (1981) 23:509) and β-globin mRNA elements can increase mRNA

stability, whereas certain AU-rich sequences in mRNA can decrease mRNA stability (Shyu *et al.*, Genes and Development (1989) 3:60). In addition, AU regions in 3' non-coding regions may be used to destabilize mRNA if a short half life mRNA is desirable. A 3'-intron should be avoided, particularly a SV40 3'-intron. If used, the 3'-intron should be greater than about 70 bp.

The nucleic acid construct may include sequences for selection, such as a neomycin resistance gene, dihydrofolate reductase gene, and/or signal sequences to regenerate recombinant proteins that are targeted to different cellular compartment or secreted when the wild type sequence is not. Any of a variety of signal sequences may be used which are well known to those skilled in the art. The signal sequences may allow generation of new vaccine strategies or produce soluble antagonists directly against specific cell surface receptors such as transformed oncogenes. The sequences for selection may be on a separate plasmid and cotransfected with the plasmid carrying the nucleic acid coding for the therapeutic polypeptide. The selection plasmid may be complexed to a different carrier or to the same carrier as the therapeutic plasmid.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences. Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

It may be desirable to produce mutants or analogs of the proteins of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution

of one or more nucleotides within the sequence. The mutation can be one that affects secretion of a normally secreted protein, so as to eliminate or decrease systemic side effects of the protein, for example, tumor necrosis factor. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., Sambrook *et al.*, infra; *DNA Cloning*, Vols. I and II, supra; *Nucleic Acid Hybridization*, infra.

If the gene sequence of the desired protein is not known, it can be obtained using the following general techniques. The desired protein can be isolated from, for example, tissue samples containing the same. This is generally accomplished by first preparing a crude extract which lacks tissue components and several extraneous proteins. The desired proteins can then be further purified i.e. by column chromatography, HPLC, immunoabsorbent techniques or other conventional methods well known in the art. Purification of the protein permits the sequencing of the same by any of the various methods known to those skilled in the art. For example, the amino acid sequences of the subject proteins can be determined from the purified proteins by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art.

Once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen DNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., *DNA Cloning*: Vol. I, supra; *Nucleic Acid Hybridization*, supra; *Oligonucleotide Synthesis*, supra; Sambrook, *et al.*, supra. First, a DNA library is prepared. The library can consist of a genomic DNA library from the species of choice. Once the library is constructed, oligonucleotides to probe the library are prepared and used to isolate the gene encoding the desired protein. The oligonucleotides are synthesized by any appropriate method. The particular nucleotide sequences selected are chosen so as to correspond to the codons encoding a known amino acid sequence from the desired protein. Since the genetic code is degenerate, it will often be necessary to

synthesize several oligonucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of the protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate.

In certain circumstances, one of skill in the art may find it desirable to prepare probes that are fairly long, and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in corresponding nucleic acid sequences, particularly if this lengthy and/or redundant region is highly characteristic of the protein of interest. It may also be desirable to use two probes (or sets of probes), each to different regions of the gene, in a single hybridization experiment. Automated oligonucleotide synthesis has made the preparation of large families of probes relatively straight-forward. While the exact length of the probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

The selected oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin using standard procedures. The labeled set of probes is then used in the screening step, which consist of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors, such as the length of the probe and whether the probe is derived from the same species as the library, or an evolutionary close or distant species. The selection of the appropriate conditions is within the skill of the art. See, generally, *Nucleic Acid Hybridization*, supra. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; i.e., hybridization is due to a sufficient degree of nucleic acid homology (e.g., at least about 75%), as opposed to nonspecific binding. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a gene for the desired protein. The desired DNA sequence can then be cloned into a cloning vector and further used, as described below.

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Preparation of Lipid carriers

Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic lipid carriers are particularly preferred because a tight charge complex can be formed between the cationic lipid carrier and the polyanionic nucleic acid, resulting in a lipid carrier-nucleic acid complex which will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells after the aerosolized DNA:lipid carrier complex has been deposited in the lung. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone, *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs, *et al.*, *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Lipid carriers can be prepared from a variety of cationic lipids, including DOTAP, DOTMA, DDAB, L-PE, and the like. Lipid carriers containing a cationic lipid, such as {N(1-2-3-dioleyloxy) propyl}-N,N,N-triethylammonium} (DOTMA), dimethyl dioctadecyl ammonium bromide (DDAB), or 1, 2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) or lysinylphosphatidylethanolamine (L-PE) and a second lipid, such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Chol), are of particular interest. DOTMA synthesis is described in Felgner, *et al.*, Proc. Nat. Acad. Sciences, (USA) (1987) 84:7413-7417. DOTAP synthesis is described in Stamatatos, *et al.*, *Biochemistry* (1988) 27:3917. DOTMA:DOPE lipid carriers in the form of liposomes can be purchased from, for example, BRL. DOTAP:DOPE liposomes can be purchased from Boehringer Mannheim. Cholesterol and DDAB are commercially available from Sigma Corporation. DOPE is commercially available from Avanti Polar Lipids. DDAB:DOPE can be purchased from Promega. Biodegradable lipid carriers are of particular interest. Cationic lipids wherein the positive change is positioned very close to the lipid bilayer e.g. DDAB, rather than projecting out from the bilayer and therefore more exposed e.g. ethylphosphatidylethanolamine (E-PC), are also of interest.

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Cationic lipid carrier:DNA complexes are internalized by cells by a classical receptor-mediated endocytosis using cell surface receptors which contain specific binding sites for, and are able to internalize, cationic molecules. Using agents such as cytokines, growth factors, other soluble proteins and certain drugs, it is thus possible to selectively up or down regulate these cation-binding receptors. The rate of up or down regulation of these receptors by the appropriate agent will allow selection of specific cells for enhanced or reduced levels of transfection *in vivo*. Thus, the use of specific cationic lipids can confer specific advantages for *in vivo* delivery. For example, iv injection of DOTAP-containing or ethylphosphatidylcholine (E-PC) lipid carriers can target transgene expression primarily to the lung and may offer increased advantages for aerosolized delivery. Furthermore, E-PC and DOTAP, as well as L-PE and CEBA are fully metabolized by cells, whereas DOTMA cannot be fully metabolized by cells. Therefore, DOTAP and L-PE, but not DOTMA, are suitable for repeated administration to mammalian hosts. Additionally, complexing the cationic lipid with a second lipid, primarily either cholesterol (0-50 mole percent, preferably 33-50 mole percent) or DOPE can maximize transgene expression *in vivo*. For example, mixing cholesterol instead of DOPE with DOTAP, DOTMA, or DDAB may substantially increase transgene expression *in vivo*.

Particular cells within the lung may be targeted by modifying the lipid carriers to direct them to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors may be employed, to target a cell associated with a particular surface protein. A particular ligand or antibody may be conjugated to the lipid carrier in accordance with conventional ways, either by conjugating the site-directing molecule to a lipid for incorporation into the lipid bilayer or by providing for a linking group on a lipid present in the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art. For example, ligand-directed DNA-polycation complexes have been shown to transfect hepatocytes in the liver after iv injection. More precise intrapulmonary targeting may be achieved by a) altering aerosol particle size to preferentially direct the aerosol to alveoli or proximal versus distal airways or (b) to covalently couple monoclonal

antibodies to the lipid carrier surface, thereby targeting lung cells expressing the corresponding cell surface antigen or using specific cationic lipids that have specific affinities for specific lung cell types.

Non-cationic liposomes, particularly pH sensitive liposomes, offer another potentially attractive approach to *in vivo* gene therapy. However, as compared to cationic liposomes, pH sensitive liposomes are less efficient in capturing DNA and delivering DNA intracellularly. Anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidylcholine, cholesterol, phosphatidylethanolamine, dioleoylphosphatidylcholine (DOPC), dioleoylphoshatidylethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

Unexpectedly, the lipid composition of the lipid carriers used for nebulization can dramatically affect the level of transgene expression produced *in vivo*. Thus, the liposomal lipid compositions generally have a composition of 50% molar ratio of cationic lipid to non-cationic lipid, but may range from 5% to 100%. The diameter of the lipid carriers should generally be within the range of 100 nm to 10 microns, preferably 100 nm to 500 nm. Other DNA sequences, such as adenovirus VA genes can be included in the lipid carrier-DNA complex formulation or be co-transfected with the gene of interest. The presence of genes coding for the adenovirus VA gene product may significantly enhance the translation of mRNA transcribed from the plasmid.

The lipid carriers can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). MLV and LUV are prepared by vortexing rather than sonicating after addition of the aqueous material to the dry lipid film. If desired, the resulting lipid carriers can be extruded under high pressure through sized polycarbonate membranes to achieve more uniform size distributions. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of unilamellar lipid carriers. Commonly used methods for making lipid carriers include Ca^{2+}-EDTA

chelation (Papahadjopoulos, *et al.*, *Biochim. Biophys. Acta* (1975) 394:483; Wilson, *et al.*, *Cell* (1979) 17:77); ether injection (Deamer, D. and Bangham, A., *Biochim. Biophys. Acta* (1976) 443:629; Ostro, *et al.*, *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley, *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); detergent dialysis (Enoch, H. and Strittmatter, P., *Proc. Natl. Acad. Sci. USA* (1979) 76:145) and reverse-phase evaporation (REV) (Fraley, et al., *J. Biol. Chem.* (1980) 255:10431; Szoka, F. and Papahadjopoulos, D., *Proc. Natl. Acad. Sci. USA* (1978) 75:145; Schaefer-Ridder, *et al.*, *Science* (1982) 215:166).

The various lipid carrier-nucleic acid complexes wherein the lipid carrier is a liposome are prepared using methods well known in the art. See, e.g., Straubinger *et al.*, in ***Methods of Immunology*** (1983), Vol. 101, pp. 512-527. By "lipid carrier-nucleic acid complex" is meant a nucleic acid sequence as described above, generally bound to the surface of a lipid carrier preparation. The lipid carrier preparation can also include other substances, such as enzymes necessary for integration, transcription and translation, cofactors, etc. Furthermore, the lipid carrier-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types.

To prepare the complexes, generally, the nucleic acid material is added to a suspension of preformed MLVs or SUVs only after the lipid carriers have been prepared and then vortexed. When using lipid carriers containing cationic lipids, the dried lipid film is resuspended in an appropriate mixing solution such as sterile water or an isotonic buffer solution such as 10mM Tris/NaCl, or 5% dextrose in sterile water, sonicated, and then the preformed lipid carriers are mixed directly with the DNA. The lipid carrier and DNA form a very stable complex due to binding of the negatively charged DNA to the cationic lipid carriers. SUVs find use with small nucleic acid fragments as well as large regions of DNA ($\geq$ 250 kb). In preparing the lipid carrier-nucleic acid complexes, care should be taken to exclude any compounds from the mixing solution which may promote the formation of aggregates of the lipid carrier-nucleic acid complexes. Large particles generally will not be aerosolized by the nebulizer and even if aerosolized would be too large to penetrate beyond the large airways. Aggregation of the lipid carrier-nucleic acid complex is prevented by controlling the ratio of DNA to lipid

carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution, usually less than 5 mg DNA/8 ml solution, and the avoiding chelating agents as EDTA, and significant amounts of salt which tend to promote macroaggregation. The preferred excipient is water, dextrose/water or another solution having low or no ionic strength. Further, the volume must be adjusted to the minimum for deposition in the lungs of the host mammal, but taking care not to make the solution too concentrated so that aggregates form.

The choice of lipid carriers and the concentration of lipid carrier-nucleic acid complexes thus involves a two step process. The first step is to identify lipid carriers and concentration of lipid carrier-nucleic acid complexes that do not aggregate when the components are combined or during the significant agitation of the mixture that occurs during the nebulization step. The second step is to identify among those that are identified as of interest at the first step (i.e. do not aggregate) those complexes that provide for a high level of transfection and expression of a gene of interest in target cells in the lung. The level of expression and the cell types in which expression of the recombinant gene is obtained may be determined at the mRNA level and/or at the level of polypeptide or protein. Gene product may be quantitated by measuring its biological activity in tissues. For example, enzymatic activity can be measured by biological assay or by identifying the gene product in transfected cells by immunostaining techniques such as probing with an antibody which specifically recognizes the gene product or a reported gene product present in the expression cassette.

As an example, a reporter gene CAT (which encodes chloramphenicol acetyl transferase) can be inserted in the expression cassette and used to evaluate each lipid carrier composition of interest. The DNA:lipid carrier complexes must be mixed in solutions which do not themselves induce aggregation of the DNA:lipid carrier complexes such as sterile water. The expression cassette (DNA) is mixed together with the lipid carriers to be tested in multiple different ratios, ranging as an example from 4:1 to 1:10 (micrograms DNA to nanomoles cationic lipid). The results provide information concerning which ratios result in aggregation of the DNA:lipid carrier complexes and are therefore not useful for use in vivo, and which complexes remain in a form suitable for aerosolization.

The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confer the highest level of transgene expression *in vivo*. For example, the optimal DNA:lipid carrier ratios for SUV for DOTMA/DOPE, E-PC:cholesterol, ethyldimystrylphosphatidyl-choline and DDAB:Chol are 1:1 or 1:2.

Administration

The mammalian host may be any mammal having symptoms of a genetically-based disorder. Thus, the subject application finds use in domestic animals, feed stock, such as bovine, ovine, and porcine, as well as primates, particularly humans. In the method of the invention, transformation *in vivo* is obtained by introducing a non-integrating therapeutic plasmid into the mammalian host complexed to a lipid carrier, particularly a cationic lipid carrier more particularly, for human use or for repeated applications a biodegradable lipid carrier. For introduction into the mammalian host any physiologically acceptable medium may be employed for administering the DNA or lipid carriers, such as deionized water, 5% dextrose in water, and the like. Other components may be included in the formulation such as stabilizers, biocides, etc, providing that they meet the criteria outlined above, i.e. do not cause aggregation of the complexes. The various components listed above find extensive exemplification in the literature and need not be described in particular here.

The lipid carrier-nucleic acid complex is aerosolized by any appropriate method. For use with humans or other primates, the aerosol is generated by a medical nebulizer system which delivers the aerosol through a mouthpiece, facemask, etc. from which the mammalian host can draw the aerosol into the lungs. Various nebulizers are known in the art and can be used in the method of the present invention. See, for example, Boiarski, *et al.*, U.S. Patent No. 4,268,460; Lehmbeck, *et al.*, U.S. Patent No. 4,253,468; U.S. Patent No. 4,046,146; Havstad, *et al.*, U.S. Patent No. 3,826,255; Knight, *et al.*, U.S. Patent No. 4,649,911; Bordoni, *et al.*, U.S. Patent No. 4,510,829. The selection of a nebulizer system depends on whether alveolar or airway delivery (i.e., trachea, primary, secondary or tertiary bronchi, etc.), is desired as well as

verifying that the specific nebulizer system does not denature or inactivate the nucleic acid construct.

A convenient way to insure effective delivery of the nucleic acid to the alveoli is to select a nebulizer which produces sufficiently small particles for example, particles with a mean particle diameter of less than 5.0 microns (μm). More preferably the particles have a mean particle diameter of about 0.2 to about 4.0 μm, and most preferably the particles have mean diameter of about 0.2 to about 2 μm, since larger particles ($\geq$ 5 μm) are generally deposited in the proximal airways or nasopharynx. As an alternative to selecting small mean particle diameters to achieve substantial distal airway and alveolar deposition, a very high dosage of the lipid carrier-nucleic acid preparation can be administered, with a larger mean particle diameter. A proviso to such an approach is that the particular lipid carrier-nucleic acid complex is chosen that is not too irritating at the required dosage and that there be a sufficient number of particles in the total particle population having a diameter in the 0.5 to about 5 μm range to allow for deposition in the alveoli. For proximal airway delivery, the mean particle size will be larger. For example, suitable mean particle diameters will generally be less than about 15 μm, more preferably from about 4 μm, and most preferably from about 5 μm to about 10 μm.

Examples of nebulizers useful for alveolar delivery include the Acorn 1 nebulizer, and the Respirgard II® Nebulizer System, both available commercially from Marquest Medical Products, Inc., Inglewood, CO. Other commercially available nebulizers for use with the instant invention include the UltraVent® nebulizer available from Mallinckrodt, Inc. (Maryland Heights, MO); the Wright nebulizer (Wright, B.M., *Lancet* (1958) 3:24-25); and the DeVilbiss nebulizer (Mercer *et al.*, *Am. Ind. Hyg. Assoc. J.* (1968) 29:66-78; T.T. Mercer, *Chest* (1981) 80:6 (Sup) 813-817). Nebulizers useful for airway delivery include those typically used in the treatment of asthma. Such nebulizers are also commercially available. One of skill in the art can determine the usefulness of a particular nebulizer by measuring the mean particle size generated thereby with for example, a 7 stage Mercer cascade impactor (Intox Products, Albuquerque, NM). Concentrations of the lipid carrier-nucleic acid complex from the impactor plates

can be determined by eluting the complex therefrom and assessing the optical density at an appropriate wavelength and comparing the standard curves. Results are generally expressed as mass median aerodynamic diameter ± geometric standard deviation (Raabe, *J. Aerosol Sci.* (1971) 2:289-303).

The amount of lipid carriers used will be an amount sufficient to provide for adequate transfection of cells after entry of the DNA or complexes into the lung and to provide for a therapeutic level of transcription and/or translation in transfected cells. A therapeutic level of transcription and/or translation is a sufficient amount to treat or palliate a disease of the host mammal following administration of the lipid carrier-nucleic acid complex to the host mammal's lung, particularly the alveoli or airway. Thus, an "effective amount" of the aerosolized lipid carrier-nucleic acid preparation, is a dose sufficient to effect treatment, that is, to cause alleviation or reduction of symptoms, to inhibit the worsening of symptoms, to prevent the onset of symptoms, and the like. The dosages of the present compositions which constitute an effective amount can be determined in view of this disclosure by one of ordinary skill in the art by running routine trials with appropriate controls. Comparison of the appropriate treatment groups to the controls will indicate whether a particular dosage is effective in preventing or reducing particular symptoms. Appropriate doses are discussed further below. While there is no direct method of measuring the actual amount of lipid carrier-nucleic acid complex delivered to the alveoli, bronchoalveolar lavage (BAL) can be used to indirectly measure alveolar concentrations of any expressed and secreted protein, usually 18-24 hrs after inhalation to allow clearance of the protein deposited in the larger airways and bronchi.

The total amount of nucleic acid delivered to a mammalian host will depend upon many factors, including the total amount aerosolized, the type of nebulizer, the particle size, breathing patterns of the mammalian host, severity of lung disease, concentration and mean diameter of the lipid carrier-nucleic acid complex in the aerosolized solution, and length of inhalation therapy. Thus, the amount of expressed protein measured in the alveoli may be substantially less than what would be expected to be expressed from the amount of nucleic acid present in the aerosol, since a large portion of the complex may be exhaled by the subject

or trapped on the interior surfaces of the nebulizer apparatus. For example, approximately one third of the lipid carrier-nucleic acid dose that is placed into the nebulizer remains in the nebulizer and associated tubing after inhalation is completed. This is true regardless of the dose size, duration of inhalation, and type of nebulizer used. Moreover, resuspension of the residue and readministration does not significantly increase the dose delivered to the subject; about one third remains in the nebulizer. Furthermore, even with minimization of airway deposition, a portion of the dose is still deposited in the airways. Additionally, efficiency of expression of the encoded protein will vary widely with the expression system used.

Despite the interacting factors described above, one of ordinary skill in the art will be able readily to design effective protocols, particularly if the particle size of the aerosol is optimized. Based on estimates of nebulizer efficiency, an effective dose delivered usually lies in the range of about 1 mg/treatment to about 500 mg/treatment, although more or less may be found to be effective depending on the subject and desired result. It is generally desirable to administer higher doses when treating more severe conditions. Generally, the nucleic acid is not integrated into the host cell genome, thus if necessary, the treatment can be repeated on an ad hoc basis depending upon the results achieved. If the treatment is repeated, the mammalian host is monitored to ensure that there is no adverse immune response to the treatment. The frequency of treatments depends upon a number of factors, such as the amount of lipid carrier-nucleic acid complex administered per dose, as well as the health and history of the subject. As used herein, with reference to dosages, "lipid carrier-nucleic acid aerosol" refers to the amount of lipid carrier-nucleic acid complex that is placed in the nebulizer and subjected to aerosolization. The "amount nebulized" or "amount aerosolized" of the complex means the amount that actually leaves the apparatus as an aerosol, i.e., the amount placed into the apparatus less the amount retained in the reservoir and on the inner surfaces of the apparatus at the conclusion of a treatment session.

In particular treatment regimens, for example, in the treatment of cancer, it may be necessary to administer sequential doses at intervals ranging

from every 8 to 12 hours to once a month. The frequency of treatment can be monitored by evaluating whether there has been until significant amelioration or complete disappearance of the cancer with a particular treatment regimen. Treatment may have to be modified if dose-limiting host toxicity develops. Similar administration protocols also may be developed in for example, patients where all macroscopic evidence of tumor has been removed, in order to prevent tumor recurrence due to persistence of undetected microscopic disease.

To treat pulmonary infections such as bronchitis and pneumonia, it will usually be necessary to administer at least one dose per day over a period of about 4 to about 21 consecutive days or longer. The treatment is usually carried out on consecutive days because new areas of the lungs open up to penetration and deposition of the nucleic acid with increasing resolution of the infection. The success of the treatment can be monitored and the administration regimen altered by assessing conventional clinical criteria; e.g., clearing of radiographic infiltrate, improved arterial PO_2 (e.g., >70 mmHg), reduction in dyspnea, respiratory rate and/or fever. For the treatment of genetic disorders, such as cystic fibrosis, the lipid carrier-nucleic acid complex will be administered at regular intervals, from once a week to once every one to several months, in order to provide wild-type CFTR protein in critical host airway cells, since these cells continue to turn over. It may also be possible to stably transfect the CFTR gene into appropriate lung stem cells, which would then provide a continuous source of normal airway cells without requiring lifelong treatment. Potential therapeutic effects of the gene product can be measured, by determining the effects of gene expression on survival of transgenic host mammals in which the transgene is expressed. Production of significant amounts of a transgene product will substantially prolong the survival of the affiliated host.

Where expression of the polypeptide/protein or even the mRNA itself confers a changed biochemical phenotype upon the host, the presence of a new phenotype or absence of an old phenotype may be evaluated; for example, as a result of transformation of the host cells, there may be enhanced production of pre-existing desirable products formerly produced in insufficient quantities or there may be reduction or even suppression of an undesirable gene product using

antisense, ribozyme or co-suppression technologies; in the case of reduction or suppression, a reduction or elimination of the gene product may be determined.

The potential toxicity of the treatment may be evaluated by behavioral manifestations, and where appropriate, by analysis of biopsy specimens. Thus, behavioral activity which evidences distress, such as changes in activity level, changes in eating and drinking patterns and the like, can be monitored, as well as evidence of necrosis, edema or inflammation in biopsy specimens.

The subject compositions can be provided for use in one or more procedures. Kits will usually include the DNA either as naked DNA or complexed to lipid carriers. Additionally, lipid carriers may be provided in a separate container for complexing with the provided DNA. The DNA or the lipid carrier/DNA complexes may be present as concentrates which may be further diluted prior to use or they may be provided at the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in sterilized containers suitable for use with a nebulizer, so that the physician or veterinarian may employ the containers directly with a nebulizer, where the containers will have the desired amount and concentration of agents. Thus, the kit may have a plurality of containers containing the DNA or the DNA/lipid carrier complexes in appropriate proportional amounts, and optionally, appropriate diluent and mixing solutions. When the containers contain the formulation for direct use, usually there will be no need for other reagents for use with the method.

Uses

Uses of the subject invention include but are not limited to the following. The present invention is particularly useful for the delivery of substances directly into the lung for the prevention and/or treatment of pulmonary disorders such as lung cancer, emphysema, asthma, lung infections such as chronic bronchitis and pneumonia, degenerative diseases of the lung, as well as genetic disorders such as cystic fibrosis and α-1 antitrypsin deficiency.

For the treatment of lung tumors, genes encoding toxic peptides (for example, therapeutic agents such as ricin, diphtheria toxin and cobra venom

factor), wild-type tumor suppressor genes (such as p53, genes coding for mRNA sequences which are antisense to transforming oncogenes, or other antineoplastic peptides, such as tumor necrosis factor (TNF) and other cytokines, or transdominant negative mutants of transforming oncogenes), can be inserted into the nucleic acid construct and using the above described methods, complexed to a lipid carrier and delivered for expression at or near the tumor site. Some tumors such as colon tumors can be specifically targeted by incorporating targeting agents, such as antibodies directed against tumor cell surface antigens, onto the lipid carrier. See, e.g., Laserman, *et al.*, *Nature* (1980) 288:602-604; Huang, *et al.*, *Biochemistry* (1981) 20:4299-4238, for methods of incorporating antibodies onto liposomal surfaces. Similarly, genes coding for peptides known to display antiviral and/or antibacterial activity, or stimulate the host' immune system, can also be administered to the lung in order to treat pulmonary infections. Thus, the genes encoding many of the various cytokines (or functional fragments thereof), such as the interleukins, interferons, and colony stimulating factors, will find use with the instant invention. The gene sequences for a number of these substances are known. Interferon-γ produces significant anti-pneumocystis carinii pneumonial (PCP) activity in immunodeficient mice with PCP following aerosol delivery of the peptide. Beck *et al.*, *Infect. and Immun.* (1991) 59:3859-3862.

Genes encoding antioxidants will also find use for the treatment or prevention of lung damage due to degenerative lung disorders caused by smoking and other environmental agents. For example, genes encoding superoxide dismutase (SOD) or catalase, as well as α-1 antitrypsin, will be particularly useful for this purpose. These gene sequences are known. See, e.g., Long *et al.*, *Biochem*, (1984) 23:4828-4837 for the α-1 antitrypsin gene sequence. For the treatment of genetic disorders, such as cystic fibrosis and emphysema, functional genes, corresponding to genes known to be deficient in the particular disorder, can be administered to the subject. For example, it is known that individuals lacking sufficient levels of α-1 antitrypsin are prone to emphysema and other pulmonary disorders. Thus, this gene can be administered prophylactically, as well as in response to clinical manifestations of the disease, for both the prevention and/or treatment of this disorder. Similarly, the gene involved in cystic fibrosis has been

identified. Goodfellow, P., *Nature* (1989) 341:102-103; Rommens, *et al.*, *Science* (1989) 245:1059-1054; Beardsley, *et al.*, *Sci. Am.* (1989) 261:28-30. Thus, this gene, or fragments which encode a biologically active expression product, can be delivered to a mammalian host suffering from this disorder.

The invention also finds use for the delivery of substances into the systematic circulation via the lung. For example, as explained above, a number of substances, such as cytokines, are toxic when administered using conventional methods of delivery. See, e.g., Debs *et al.*, *J. Immunol* (1988) 140:3482-3488. The invention allows the delivery of these substances for example, to treat cancer, as well as bacterial and viral infections, systemically. This approach already has shown promise for the treatment of extra-pulmonary cancer in humans.

The instant methods also find use in antisense therapy, for the delivery of oligonucleotides able to hybridize to specific complementary sequences, thereby inhibiting the transcription and/or translation of these sequences. Thus, DNA or RNA coding for proteins necessary for the progress of a particular disease, can be targeted, thereby disrupting the disease process. For a review of antisense therapy and oligonucleotides useful in the same, see, Uhlmann, E. and Peyman, A., *Chem. Rev.* (1990) 90:543-584.

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the present invention.

EXAMPLES

The practice of the present invention employs unless otherwise indicated, conventional techniques of cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Vols. 1-3; DNA Cloning (1985) Vols. I and II, D.N. Glover (ed.); Nucleic Acid Hybridization (1984), B.D. Hames, *et al.*, (eds.); Perbal, B., A Practical Guide to Molecular Cloning (1984); Methods in Enzymology (the series), Academic Press, Inc.; Vectors: A Survey of Molecular Cloning Vectors and Their

Uses (1987), R.L. Rodriguez, *et al.*, (eds.), Butterworths; and Miller, J.H., *et al.*, Experiments in Molecular Genetics (1972) Cold Spring Harbor Laboratory.

Up to 48 mice may be placed in the chamber at one time. The amount of the total amount of DNA-lipid carrier complex placed in the nebulizer that is delivered to the lungs of each mouse is approximately 0.02%.

Example I

Expression of chloramphenicol acetyltransferase (CAT) gene, in rodent lungs following aerosolized delivery of lipid carrier:nucleic acid complexes.

The lipid carriers used were plasmid pRSV-CAT, as described by Gorman, *et al.*, *Proc. Natl. Acad. Sci. USA* (1982) 79:6777-6781; and Juang, and Gorman, *Mol. Cell. Biol.* (1990) 10:1805-1810; a plasmid containing the CAT gene driven by the RSV long terminal repeat; and plasmid pRSV-β-gal, as described by Hazinski *et al.*, *Am. J. Respir. Cell Mol. Biol.* (1991) 4:206-209.

The pRSV-CAT plasmid was complexed to liposomes and administered to 25 gram female BALB/c mice as follows. Two mg of pRSV-CAT was mixed with 4 μmoles of DOTMA (GIBCO BRL, Grand Island, NY)/cholesterol (2:1) small unilamellar liposomes in phosphate buffered saline and then nebulized in an Acorn I nebulizer (Marquest Medical Products, Inc., Inglewood, CO) to groups of rats or mice in an Intox nose-only exposure chamber (Intox Products, Albuquerque, NM). The same procedure was followed with 0.5 mg pRSV-CAT mixed with 1.0 μmol DOTMA-cholesterol (2:1), as well as 2.0 mg pRSV-CAT alone. Two to five days later, animals were sacrificed and lungs collected. Lungs were also collected from untreated controls. The lungs were homogenized and cells disrupted with three freeze-thaw cycles. CAT activity in aliquots from the lung extracts was measured using a standard assay as described by Wolff, *et al.*, *Science* (1990) 247:1465-1468. As can be seen in Figure 1, animals administered 2.0 mg RSV-CAT with 4.0 μmol DOTMA/cholesterol (2:1) expressed the CAT protein in the lungs while the control animals as well as animals receiving aerosolized RSV-CAT DNA alone, or lower doses of RSV-CAT:DOTMA:chol complexes did not. A similar procedure was followed with respect to pRSV-β-gal, with the exception that 50 mg of pRSV-β-gal was mixed

with 50 μmoles of DOTMA/cholesterol (2:1). The presence of β-gal activity was determined using a standard histochemical staining procedure. β-gal activity was present in the airway epithelial cells of exposed rats.

Also tested was a plasmid containing the CAT gene driven by the CMV promoter. This plasmid was made as described in Huang, M.T.F. and Gorman, C.M. *Nuc. Acids Res.* (1990) 18:937-947, with the exception that a CMV promoter and a hybrid intron sequence were used rather than the SV40 promoter in the plasmid pML.I.CAT, described therein. Briefly, the CAT lipid carrier was constructed by first making a pML-based plasmid containing the CMV promoter immediately followed by a portion of the 5'-untranslated leader from the adenovirus-major late (AML) region. This region contained all but the first 13 nucleotides of the first exon of the tripartite leader plus a portion of an intervening sequence (IVS) from the AML region. A synthetic oligonucleotide was inserted which merged with the adenovirus intron to provide a functional splice acceptor sequence derived from an IgG variable region. Bothwell, *et al.*, *Cell* (1981) 24:625-637. This plasmid was then cut at two restriction sites bordering the intron (*Cla*I and *Pst*I) to remove a 292 bp fragment. A matching synthetic oligonucleotide linker was inserted. The plasmid was termed pCIS-CAT.

To test for expression of the CAT gene using pCIS-CAT, 12 mg pCIS-CAT was mixed with 24 μmoles of DOTMA/DOPE (1:1). Female ICR mice were placed in three different aerosol receiving chambers. All mice received the same amount of the CAT expression plasmid complexed to liposomes, as described above. Animals 1-3 were exposed to the aerosol in an Intox designed aerosol chamber. Animals 4-7 were exposed to the aerosol in a modified rat cage containing dividers for individual mice. Animals 8-10 were placed in a smaller, similarly modified mouse cage after being put in the restrainers used in the Intox chamber. 48 hours following aerosolization, the animals were sacrificed and whole lungs assayed for CAT expression using the chromatographic CAT assay. As can be seen in Figure 2, a single aerosol dose of a CAT gene-expression plasmid complexed to cationic liposomes can produce high-level transgene expression in the lungs of mice. Significant levels of transgene expression are present in the lungs of all 7 mice (numbers 1-3 and 8-10) which were exposed to

the aerosol mist in Intox nose-only exposure tubes which were constructed to maximize the amount of aerosol that the mice inhaled. The amount of variation seen here is comparable to that seen in other aerosol experiments and may have several explanations, including variations in exposure to the aerosol mist, individual variations in efficiency of nasal filtration, etc.

Example II

Aerosol administration of CMV-CAT cationic lipid carrier complexes produces high level, lung specific expression of the CAT gene.

Animals. Two month old, female, ICR mice were used in all experiments.

Preparation of plasmid DNA. The chloramphenicol acetyltransferase (CAT) gene was used as a reporter to measure transgene expression levels (Gorman *et al.*, *Proc. Nat'l Acad Sci (USA)* (1982) 79: 6777-6781). The plasmid used contains the CAT gene fused to the human cytomegalovirus (CMV) immediate early promoter-enhancer element (pCIS-CAT). The plasmid was purified using alkaline lysis and ammonium acetate precipitation (Sambrook *et al.* (1989) supra), and the nucleic acid concentration measured by UV absorption at 260 nm. The CAT gene is not present in eukaryotic cells. Its product is an enzyme which catalyzes the transfer of acetyl groups from acetylCoA to the substrate chloramphenicol.

Preparation of cationic liposomes. Liposomes were prepared as small unilamellar vesicles (approximately 100 nm in diameter) containing the cationic lipid DOTMA as DOTMA:DOPE (1:1 mole ratio). DOTMA is (N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (Syntex Corporation), and DOPE is the neutral lipid dioleoylphosphatidylethanolamine (Avanti Polar Lipids). Stock solutions of the lipids were dissolved in chloroform and stored under argon at -20°C. Lipids were mixed in a round-bottomed flask and evaporated to dryness on a rotary evaporator under reduced pressure. Double-distilled water was added to produce final lipid concentrations of 10mM each, and the resulting mix was sonicated for approximately 20 minutes in a bath sonicator (Laboratory Supplies, Hicksville, NY). The liposomes were stored under argon at 4°C until use.

Aerosol delivery of plasmid/liposome complexes to mice. Twelve mg of plasmid complexed to 24 μmols of DOTMA:DOPE (1:1 mole ratio) liposomes was aerosolized and administered to mice over two different aerosol periods on the same day. In order to prevent aggregation and precipitation of the oppositely charged components, the plasmid and the liposomes were diluted separately in sterile water prior to mixing. Six mg of plasmid DNA and 12 μmols of DOTMA:DOPE (1:1 mole ratio) liposomes were each diluted to 8 ml with water and mixed. Four ml was then placed into each of two Acorn I nebulizers (Marquest, Englewood, CO), the animals placed into an Intox small animal exposure chamber (Albuquerque, NM), and an air flow rate of 4 L min^{-1} used to generate the aerosol. Approximately 90 minutes were required to aerosolize 4 ml. The animals were removed from the chamber for 1-2 hours and then the above procedure was repeated with a second 4 ml dose.

Radiometric Assay of CAT Activity. Organs were dissected from animals sacrificed in a CO_2 chamber at periods from 1 to 21 days following aerosolization, washed in cold phosphate buffered saline (PBS), and homogenized using a hand-held tissue homogenizer in 250 mM Tris-HC1, pH. 7.5, containing 5 mM EDTA for lungs and spleen and 250 mM Tris-HC1, pH 7.5, containing 5 mM EDTA plus the protease inhibitors aprotinin, E-64, and leupeptic (Boehringer Mannheim) for liver, heart and kidneys. The inhibitors prevent degradation of acetylated chloramphenicol species generated during the assay, thereby allowing optimal detection of CAT expression.

Following homogenization of the tissue, cells were lysed by three freeze/thaw cycles, the lysate heated (65°C for 10 minutes), and centrifugated (16,000 x g, 2 minutes). The protein concentrations of the extracts were measured using a Coomassie blue-based assay (Bio-Rad). Protein concentrations were normalized and a volume of extract added to 10 μl of 100 mM acetylCoA (Sigma), 0.3 μCi of [^{14}C]-labelled chloramphenicol (Amersham), and distilled water to a final volume of 180 μl, and allowed to react at 37°C for 8-10 hours (Gorman *et al.* (1982) supra). Following the reaction, the acetylated and unacetylated chloramphenicol species were extracted with cold ethyl acetate, spotted on silica TLC plates, and developed with a chloroform:methanol (95:5v/v) solvent. The

TLC plates were exposed to photographic film (Kodak X-OMAT)* for one to three days, then evaluated by visual inspection.

Preparation of Genomic DNA and Southern Hybridization. Immediately following aerosolization, mice were sacrificed and their lungs removed. Genomic DNA was isolated and analyzed by Southern hybridization (Sambrook *et al.* (1989) supra) using a Hybond N+* membrane (Amersham). A CAT probe was prepared from a 1.6 kb fragment of the CAT gene labelled with α-[^{32}P]dATP by random priming, which yielded a probe with an approximate specific activity of 2 x 10^9dpm/μg. After hybridization, the membrane was washed three times in 2xSSC, 0.1%SDS at 65°C for 20 minutes and exposed to film for 24 hours. In order to determine the approximate transfected CAT gene copy number, blots were also hybridized with a 1.1 kb BSU 36-1 single copy probe from a mouse factor VIII-A genomic clone (Levinson *et al.*, *Genomics* (1992) 13: 862-865). Relative amounts of the CAT plasmid deposited in individual mouse lungs were quantitated by phosphorimagining analysis using a Molecular dynamics 400A phosphorimaginer* (Johnson *et al.*, *Electrophoresis* (1990) 11: 355-360). The amount of retained probe in each lane following hybridization with the CAT probe was normalized to the amount of DNA loaded per lane using the counts measured after hybridization with a Factor VIII-A single copy probe.

In *Situ* Immunochemical Staining for CAT enzyme. At selected time points following aerosolization, mice were sacrificed and their lungs immediately removed. The lungs were slowly inflated with phosphate buffered saline (PBS) containing 33% by volume OCT (Miles, Inc.), placed in a tissue cassette filled with OCT, and frozen in 2-methylbutane chilled in a dry ice/ethanol bath. Cryosections were cut at 5 μm and collected onto salinized slides. CAT was detected after fixation of cryosections for 10 minutes in either 4% acetone or 2% paraformaldehyde in PBS containing 0.1% Tween 20 (PBST). All subsequent dilutions and washes were also done in PBST.

Following fixation, sections were washed three times (5 minutes each) then covered with 10% normal rabbit serum for 10 minutes at 20°C. The serum was replaced with diluted (1:500) rabbit polyclonal antibody against CAT (Drs. Parker Antin and David Standring, UCSF Medical Center). The antibody

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covered section was gently overlaid with a siliconized coverslip and incubated in a humid chamber at 4°C for 24 hours. Slides were then warmed to 20°C and washed three times. The presence of bound rabbit antibody against CAT was detected by covering sections with biotinylated, affinity purified, goat anti-rabbit antibody (Vector Laboratories) diluted 1:300 for 1 hour, followed by washing (3 x 10 minutes) and replacement with streptavidin labelled with alkaline phosphatase (Zymed, South San Francisco) for 20 minutes. Immobilized alkaline phosphatase was detected using AP-red (Zymed) as the chromogen, with endogenous alkaline phosphatase being inhibited with levamisole (Zymed). To control for potential spurious adherence of the streptavidin conjugate to bronchiolar epithelium, some sections were treated with free avidin and biotin prior to application of the primary antibody. Other controls, run concurrently, included the use of normal rabbit serum in place of primary antibody and the use of lung tissue from untreated mice. Photo-microscopy was performed using Kodak Ektachrome 64T* film X50 (Fig. 6 A,D) and X250 (Fig. 6 B,C,E,F).

Results

Initially, mice were exposed either to an aerosol generated from a solution containing 12 mg of a CMV-CAT expression plasmid alone or to an aerosol generated from a solution containing 12 mg of CMV-CAT complexed to 24 μmoles of DOTMA:DOPE (1:1) liposomes. Aerosols were administered to animals after they were placed individually in nose-out cones and inserted into an Intox small animal exposure chamber. The mice showed no apparent ill effects or respiratory distress either during or after aerosol exposure. Figure 7 shows the results of CAT assays from extracts of the lungs of mice sacrificed 72 hours following aerosol administration. Significant CAT gene expression was seen only in mice exposed to aerosolized DNA/liposome complexes.

How long CAT protein was present in the lungs of mice and whether expression of the reporter gene was limited to the lung was also investigated. Despite inter-animal variation, high levels of CAT activity are present for at least 21 days following a single aerosol dose of DNA/liposome complexes (Fig. 8A). No CAT activity was detectable in extracts from the heart,

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spleen, kidneys or liver of animals that showed high level expression in the lung (Fig. 8B), suggesting that transgene expression following aerosol delivery is restricted to the lung. This is consistent with prior observations showing that penetration of very high molecular weight substances through the respiratory epithelium of normal animals is very limited. Plasmid DNA/liposome complexes have molecular weights greater than 10^6 daltons.

Although the small animal exposure chamber used in these experiments is designed to efficiently deliver a uniform aerosol dose to up to 48 animals, we have observed significant variations in the level of CAT activity in the lungs of mice within a single experiment. One possible explanation for this variability is that the amount of DNA/liposome complex deposited in the lungs of mice is not uniform. In order to test this hypothesis, initial lung deposition of liposomes was measured using fluorescence analysis and initial lung deposition of DNA was measured using Southern blot analysis.

Aerosolized cationic liposomes alone or DNA/liposome alone or DNA/liposome complexes containing 0.5 mole percent of a fluorescently labelled lipid, rhodamine-phosphatidylethanolamine, were administered to mice. Immediately following aerosolization, the animals were sacrificed and their lungs removed, homogenized and rhodamine fluoreacence measured using a fluorimeter. The recovered fluorescence per animal was 0.06% $\pm$ 0.02 (S.D.) of the total amount aerosolized. This suggests that less than 10 μg out of the 12 mg of DNA aerosolized per experiment was actually depotited in the lung. In addition, there was no significant difference in lipid deposition between animals receiving liposomes alone and those receiving the DNA/liposomes complexes. Since it is possible that a disruption of the complex could have occurred during nebulization, the amount of CAT gene deposited during aerosolization (Fig.9) was also assessed. Immediately following aerosol delivery of DNA/liposome complexes, mice were sacrificed and total lung DNA prepared. Southern blots were probed with $\alpha[^{32}P]$-labelled CAT gene. Labelled bands were scanned and demonstrated less than a 4-fold difference in plasmid deposition between animals in the same experiment (Fig. 9). These results suggest that the mouse to mouse variation in CAT gene levels following aerosol delivery (up to ten-fold) is not only a function of the

amount of complex initially deposited in the lung, but also may reflect differences in the site of uptake, rate of lung clearance, and/or variation in the ability of different lung cell types to express the transgene.

To determine the types and percentage of lung cells which were transfected *in vivo*, lungs of mice sacrificed 72 hours following exposure to an aerosol containing DNA/liposome complexes were cryosectioned, probed with a polyclonal anti-CAT antibody and counterstained to detect intracellular CAT protein (Fig. 6). Lung sections taken from DNA/liposome treated mice had a diffuse immunostaining pattern involving bronchiolar and alveolar components. The bronchiolar epithelial cytoplasm stained with greatest intensity and uniformity. CAT antigen was detected (as demonstrated by red staining) in nearly all conducting airways with only rare individual or 2-3 cell clusters not staining (Fig. 6 A,B). The diffuse alveolar pattern was due to moderately intense staining of the majority of alveolar lining cells (Fig. 6C). These areas occasionally faded into small, randomly scattered regions where lining cell staining was faint. Focal, intense staining (arrows) occurred in the cytoplasm of scattered, individual, alveolar lining cells (Fig. 6C). Controls included substitution of the primary antibody with normal rabbit serum (Fig. 6D) and use of lung sections from untreated animals (Fig. 6 E,F). Immunostaining was not detectable in either of the control preparations. Examination of multiple sections of lung from treated and control mice demonstrated no significant lesions which would indicate adverse effects of the aerosol treatment.

Example III

High level airway expression of the human CFTR gene in mouse lungs after aerosol administration of DDAB:cholesterol liposome-pZN32 complexes

Animals.

Two months old, female, ICR mice obtained from Simonsen, Gilroy, CA, were used.

Preparation of plasmid DNA.

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The plasmid liposome used, pZN32, contains the human CFTR gene coding region fused to the human cytomegalovirus immediate early promoter-enhancer element shown in Figures 3-5 attached hereto. A full restriction map of the immediate early enhancer and promoter region of HCMV (Towne) and HCMV (AD169) is provided in Figs. 11A and 11C. The two sequences are compared in Fig. 11B. pZN32 was purified using alkaline lysis and ammonium acetate precipitation, and the nucleic acid concentration measured by UV absorption at 260 nm.

Preparation of cationic lipid carriers.

Lipid carriers were prepared as small unilamellar vesicles (approximately 100 nm in diameter) containing the cationic lipid DDAB (dimethyl dioctadecyl ammonium bromide) as DDAB cholesterol in a 1:1 molar ratio. DDAB was purchased from Sigma, St. Louis, MO, and cholesterol was purchased from CalBioChem, San Diego, CA. Stock solutions of the lipids were dissolved in chloroform. Lipids were mixed in a round-bottomed flask and evaporated to dryness on a rotary evaporator under reduced pressure. Double distilled water was added to produce final lipid concentrations of 10 mM each, and the resulting mix was sonicated for approximately 20 minutes in a bath sonicator (Laboratory Supplies, Hicksville, NY).

Aerosol delivery of plasmid/lipid carrier complexes to mice.

Twelve mg of pZN32 complexed to 24 μmols of DDAB:cholesterol (1:1 mole ratio) liposomes was aerosolized over two different aerosol periods on the same day. To prevent aggregation and precipitation of the oppositely charged components, the liposomes and DNA were diluted separately in sterile water prior to mixing. Six mg of plasmid DNA and 12 μmols of DDAB:cholesterol (1:1 mole ratio) liposomes were each diluted to 8 ml with water and mixed. Four ml of the DNA-liposome mixture was then placed into two Acorn I nebulizers (Marquest, Englewood, CO), and the animals placed in an Intox small animal exposure chamber (Albuquerque, NM). An air flow rate of 4 L min^{-1} was used to generate the aerosol. Ninety minutes were required to aerosolize this volume (4 ml) of DNA-liposome mixture. The animals were removed from the chamber for 1-2 hours and then the above procedure was repeated with a second 4 ml dose.

Immunohistochemical staining for the human CFTR protein in mouse lungs.

At selected time points following aerosolization, mice were sacrificed and their lungs immediately removed. The lungs were slowly inflated with phosphate buffered saline (PBS) containing 3.3% by volume OCT (Miles, Inc.), then placed in a tissue cassette filled with OCT, and frozen in 2-methylbutane chilled in a dry ice/ethanol bath. Cryosections were cut at 5 μm and collected onto sialinized slides. CFTR protein was detected after fixation of cryosections for 10 minutes in either 4% acetone or 2% paraformaldehyde in PBS containing 0.1% Tween 20*(PBST). All subsequent dilutions and washes were done in PBST. Following fixation, sections were washed three times (5 minutes each) with PBST then covered with 10% normal rabbit serum for 10 minutes at 20°C. Immunolocalization of CFTR was then performed using an affinity purified rabbit polyclonal anti-CFTR antibody, α-1468, provided by Dr. Jonathan Cohn, Duke University. The serum was replaced with α-1468, diluted (1:1000). The antibody-covered section was gently overlaid with a siliconized coverslip and incubated in a humid chamber at 4°C for 24 hours. Slides were then warmed to 20°C and washed three times. The presence of bound rabbit antibody against CFTR was detected by covering sections with biotinylated, affinity-purified, goat anti-rabbit antibody (Lipid carrier Laboratories), diluted 1:300 for 1 hour, followed by washing (3 x 10 minutes) and replacement with streptavidin labelled with alkaline phosphatase (Zymed, South San Francisco) for 20 minutes. Immobilized alkaline phosphatase was detected using AP-red (Zymed) as the chromogen; endogenous alkaline phosphatase was inhibited with levamisole (Zymed). Other controls, run concurrently, included the use of normal rabbit serum in place of primary antibody and the use of lung tissue from untreated mice. Photo-microscopy was performed using Kodak Ektachrome 64T film at X50 and X250.

Results.

Photomicrographs of frozen sections (viewed at different magnifications) of mouse lung 48 hours following aerosol exposure to pZN32-DDAB:cholesterol 1:1 mole ratio liposome complexes and lung from untreated

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control are shown in Figs. 10A-10E. As demonstrated by the intense staining with the polyclonal anti-CFTR antibody, α-1468, the overwhelming majority of the airways were transfected with the human CFTR gene. See Figs. 10A, 10C and 10E. By visual inspection, essentially all the cells in transfected airways stain positively, demonstrating that the overwhelming majority of airway cells are transfected with the human CFTR gene *in vivo* with a single aerosol dose of pZN32 complexed to DDAB-cholesterol 1:1 mole ratio liposomes. Representative sections are shown in Figure 10. There was no histologic evidence of lung damage, inflammation or edema present in any of the pZN32-DDAB:cholesterol-1:1 mole ratio liposome-treated animals. pZN32-DDAB:cholesterol-1:1 mole ratio liposome-treated and control animals could not be distinguished histologically. Significant expression of the human CFTR gene is present in at least 50% of all the airways and at least 50% of all of the airway lining cells (by visual inspection) in mouse lungs for at least 60 days following a single aerosol dose of pZN32 complexed to DDAB-cholesterol 1:1 mole ratio liposomes. Frozen sections of mouse lungs from control animals (Figs. 10B and 10D) do not show any detectable staining for CFTR, confirming that all the CFTR expression present in Fig. 10A, 10C and 10E is due to transfection of lung cells with the human CFTR gene.

Example IV

Efficient transfection of a variety of human lung cancer cell lines using cationic liposome-mediated delivery of DNA.

Method:

Cell Culture: NCl-H69, NCl-H82, and NCI-H520. H69 and H520 cells were grown in RPMI-1640 with 10% fetal bovine serum (FBS) and H82 cells were grown in Dulbecco's minimum essential medium (DME)-H21 with 10% FBS.

Liposome preparation: Liposomes were prepared as follows: a total of 4 μmoles of lipid dissolved in chloroform, (or in ethanol (DOTMA)) were evaporated to dryness on a rotary evaporator. One ml of 50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 100 mM $ZnCl_2$ buffer per 20 mmoles of lipid was added, and the mixture was sonicated in a bath sonicator (Laboratory Supply Co., Hicksville, NY) for 20 min. The resulting liposomes have an approximate mean diameter of 100 ± 25

nm. The following liposome preparations were used: pure DOTMA, DOTMA:chol in a 2 to 1 molar ratio, pure L-PE or L-PE:chol-b-ala in a 6 to 4 molar ratio.

Cellular transfection: For transfection of cells, 2 x 10^6 cells in 4 ml of serum-free medium were plated in 100 mm plastic petri dishes (Falcon, Oxnard, CA). The plasmid DNA-liposome complex was prepared by first adding 1) DNA and then 2), liposomes and mixing gently. The complex was then suspended in 1 ml of serum-free medium and added to the cells. Four hours later, the cells were washed twice, resuspended in 10 ml of serum-containing medium, and subsequently harvested, either 44 hours later. Just prior to harvesting, the cells were washed 2 times, and the plates were then scraped with a rubber policeman. The cells were centrifugated at 1,000 x g for 5 min, and 0.135 ml of 0.25 M Tris buffer was added to each pellet. The cells were freeze-thawed 3x, heated at 65°C for 10 min, and spun at 12,500 x g for 10 min. The supernatant was assayed for protein and 20 μg of supernatant protein per sample was used to measure CAT activity, as described above.

Results: The ability of cationic liposomes to mediate transfection of two different human small cell lung cancer lines (H-69 and H-82) and a squamous cell lung cancer line (H-520) was assessed. All three lines were very efficiently transfected by RSV-CAT when complexed to 3 different cationic liposome formulations. These human cell lines were either as or more efficiently transfected than rodent cell lines transfected under comparable conditions.

As shown by the above results, a single aerosol dose of an expression liposome, containing a gene of interest, complexed to cationic liposomes transfects the majority of the cells lining both the conducting airways and the alveoli of the lung, the gene product is present in the lung for at least 60 days, the expression appears to be lung-specific, and there is no histological evidence of damage following exposure. Thus, the aerosolized cationic liposomes mediate efficient transfection of non-dividing as well as dividing cells. This is important because many airway epithelial cells are well differentiated and divide slowly or not at all. The lipid carriers appear to be both well tolerated and non-immunogenic. Additionally, the effects of repeated aerosol administration of the DNA/liposome complexes is effective and is non-toxic. The cationic liposome-mediated DNA delivery by aerosol provides high level, lung-specific transgene expression *in vivo*.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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CLAIMS:

1. A composition comprising:

a nebulized transfection agent, prepared by nebulizing a mixture comprising complexes between transcription cassettes or expression cassettes and cationic lipid carriers in a pharmaceutically acceptable carrier or diluent, wherein said mixture is substantially free of macroaggregates of said complexes and said lipid carriers have a diameter from 0.1 microns to 10 microns and the ratio of DNA to lipid carrier is in a ratio of 4:1 to 1:10 micrograms DNA to nanomoles cationic lipid and wherein said transcription cassettes or expression cassettes comprise a DNA sequence capable of producing a transcription product in a mammalian cell transfected by said nebulized transfection agent.

2. The composition according to claim 1, wherein said DNA sequence comprises an inducible promoter.

3. The composition according to claim 2, wherein said inducible promoter is a cell specific promoter, a tissue specific promoter or a hormone responsive promoter.

4. The composition according to claim 2, wherein said DNA sequence comprises an SV40 enhancer element whereby transcription from said promoter is enhanced.

5. The composition according to any one of claims 1 to 4, wherein said cationic lipid carrier comprises a lipid selected from the group consisting of N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA); dimethyldioctadecylammoniumbromide (DDAB); 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP); lysinylphosphatidyl-

ethanolamine (L-PE); dioleoylphosphatidylethanolamine (DOPE); and cholesterol (Chol).

6. The composition according to any one of claims 1 to 4, wherein said cationic lipid carrier comprises cholesterol and a lipid selected from the group consisting of N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA); dimethyldioctadecylammoniumbromide (DDAB); 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP); and lysinylphosphatidyl-ethanolamine (L-PE).

7. The composition according to any one of claims 1 to 4, wherein said cationic lipid carrier comprises dioleoylphosphatidylethanolamine (DOPE) and a lipid selected from the group consisting of N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA); dimethyldioctadecylammoniumbromide (DDAB); 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP); and lysinylphosphatidyl-ethanolamine (L-PE).

8. The composition according to any one of claims 1 to 7, for use in the treatment or therapy of a human or animal body, said human or animal body having an aberrant expression of said transcription product.

9. The composition for use according to claim 8, wherein said use is use for inhibiting growth of tumor cells in a mammal.

10. The composition for use according to claim 8, wherein said use is use for providing or enhancing production of a polypeptide in cells in lungs of a mammal.

11. The composition for use according to claim 10, wherein said cells are distal airway cells or proximal airway cells.

12. The composition for use according to claim 10, wherein said cells are tracheal cells.

13. The composition for use according to any one of claims 10 to 12, wherein said cells are normal cells.

14. The use of a composition as defined in any one of claims 1 to 7 for the manufacture of a medicament for the treatment of a human or animal body, said human or animal body having an aberrant expression of said transcription product.

15. The composition according to any one of claims 1 to 4, wherein said transcription cassettes or expression cassettes and said cationic lipid carriers are present in said mixture in a ratio in the range of from about 4:1 to about 1:10 micrograms DNA to nanomoles of cationic lipid.

16. The composition according to any one of claims 1 to 4, wherein said cationic lipid carriers are small unilamellar vesicles.

17. The composition according to claim 16, wherein said small unilamellar vesicles comprise (a) dioleoylphosphatidylethanolamine (DOPE) and N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) or (b) dimethyldioctadecyl-ammoniumbromide (DDAB) and cholesterol.

18. The composition according to any one of claims 1 to 4, wherein said transcription cassettes or expression cassettes and said cationic lipid carriers are present in said mixture in a ratio in the range of from about 1:1 to 1:2 micrograms DNA to nanomoles of cationic lipid.

19. A composition for use according to claim 8, wherein said use is use for altering the phenotype of cells of a lung of a mammal.

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20. A kit for use in the treatment or therapy according to any one of claims 8 to 14, said kit comprising in combination:

a container containing said transcription cassettes or expression cassettes;

another container containing a specific amount of cationic lipid carriers; and

instructions.

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PATENT AGENTS

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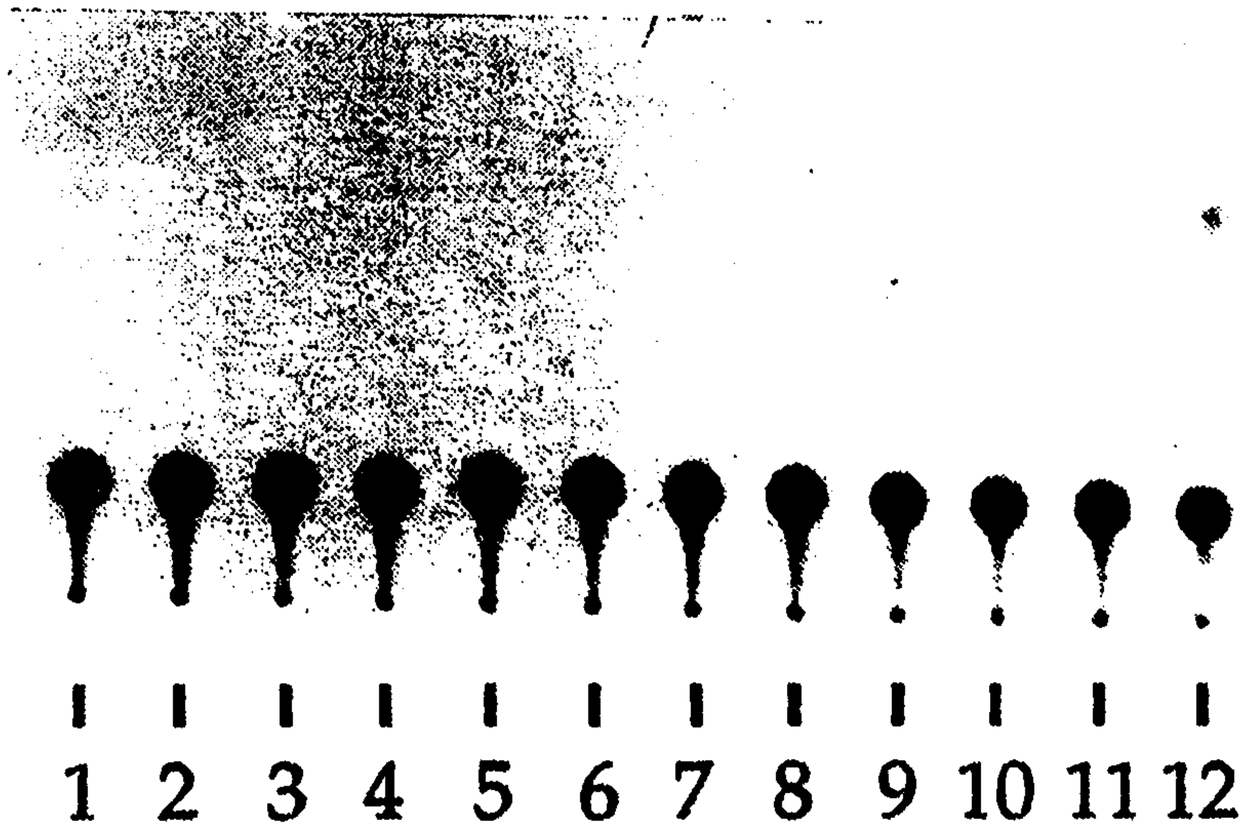

FIGURE 1

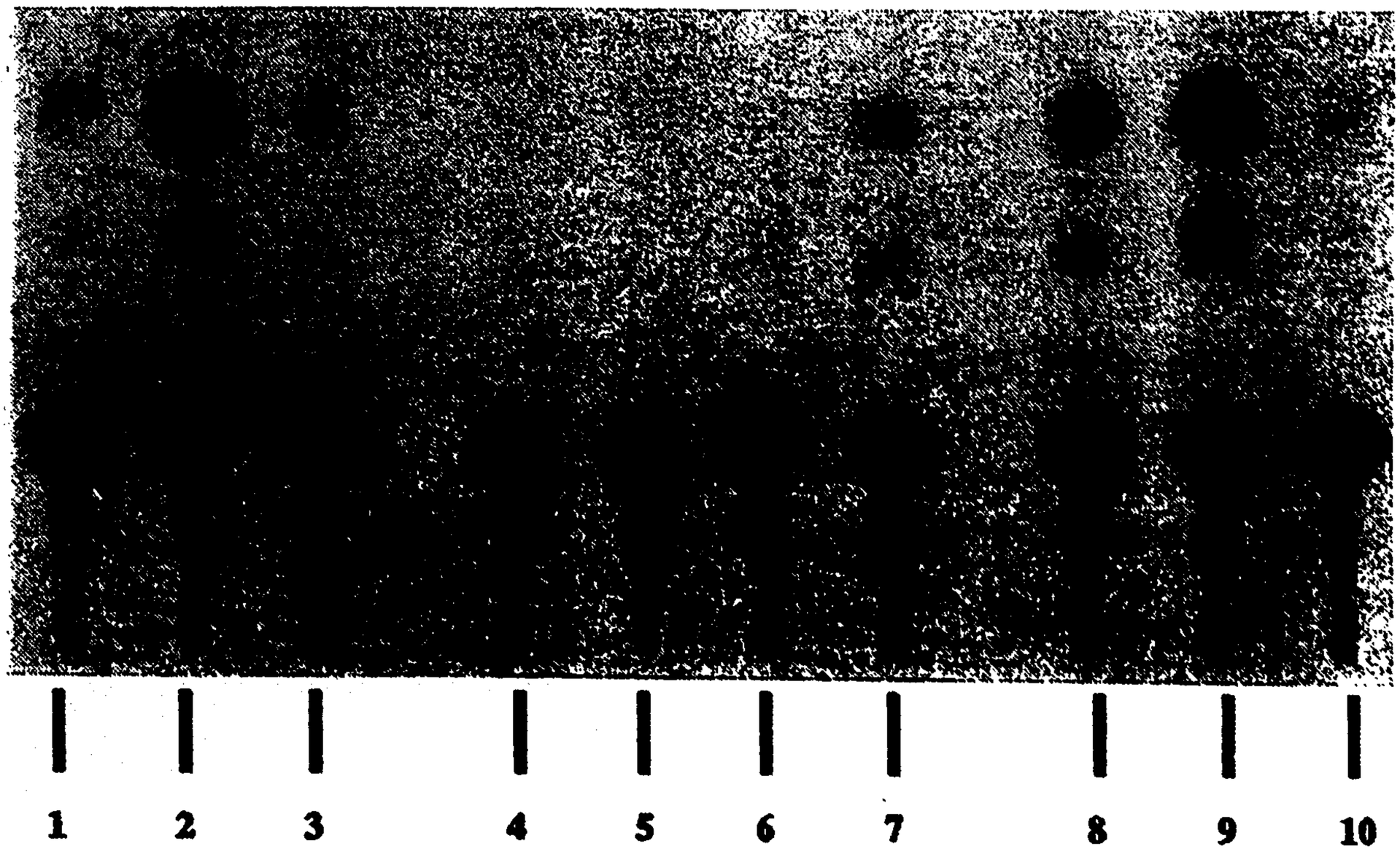

FIGURE 2

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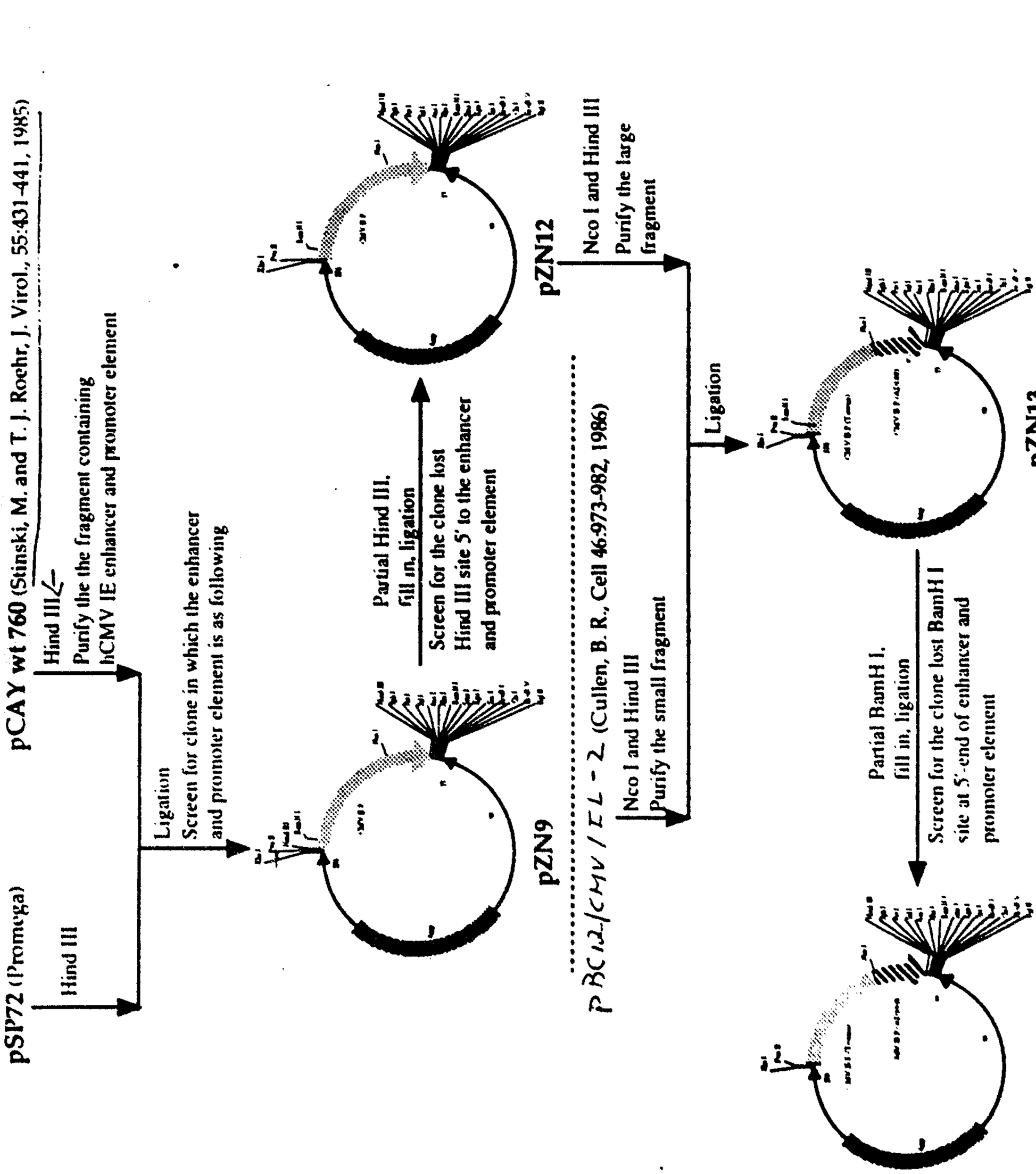

FIGURE 3

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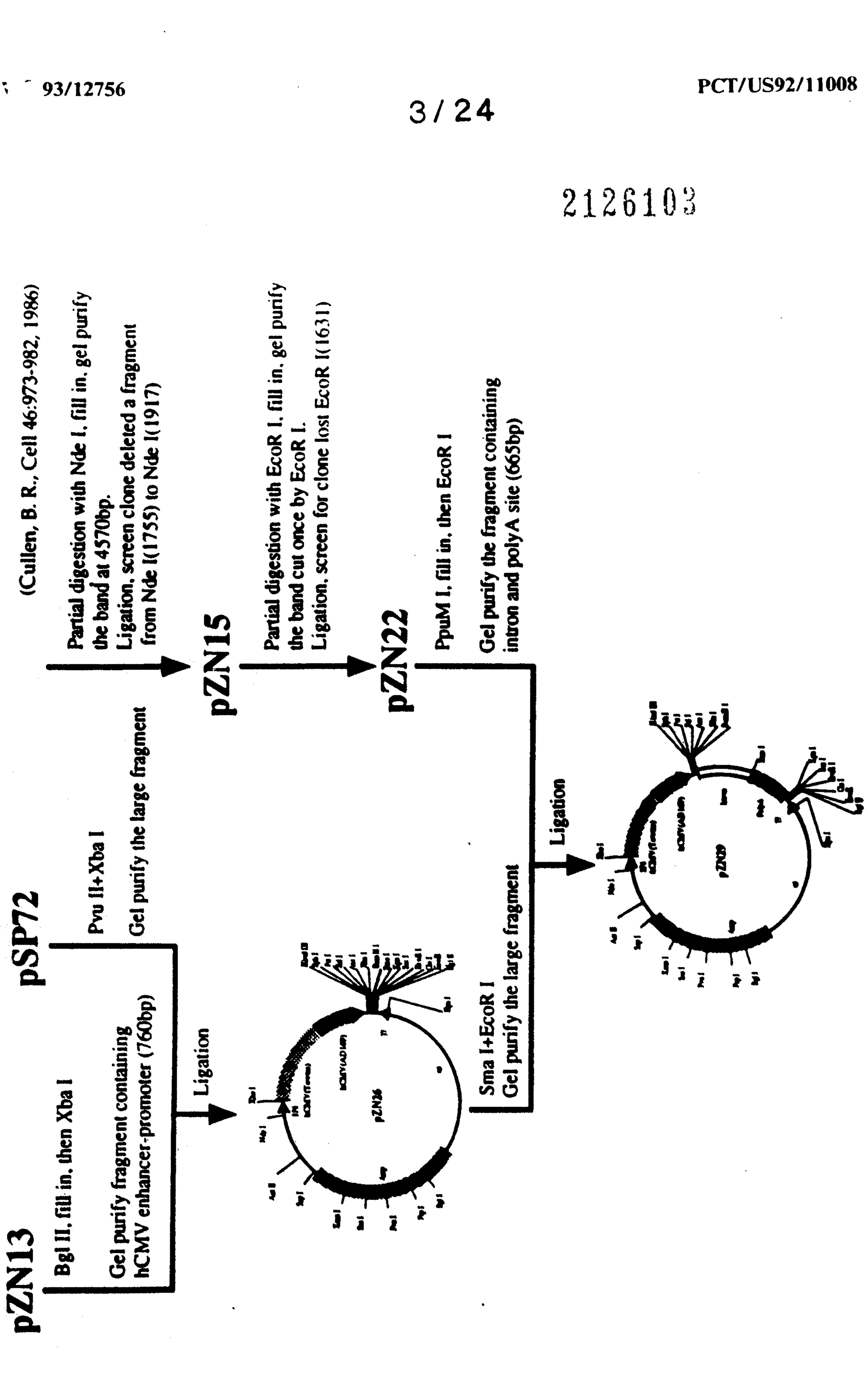

FIGURE 4

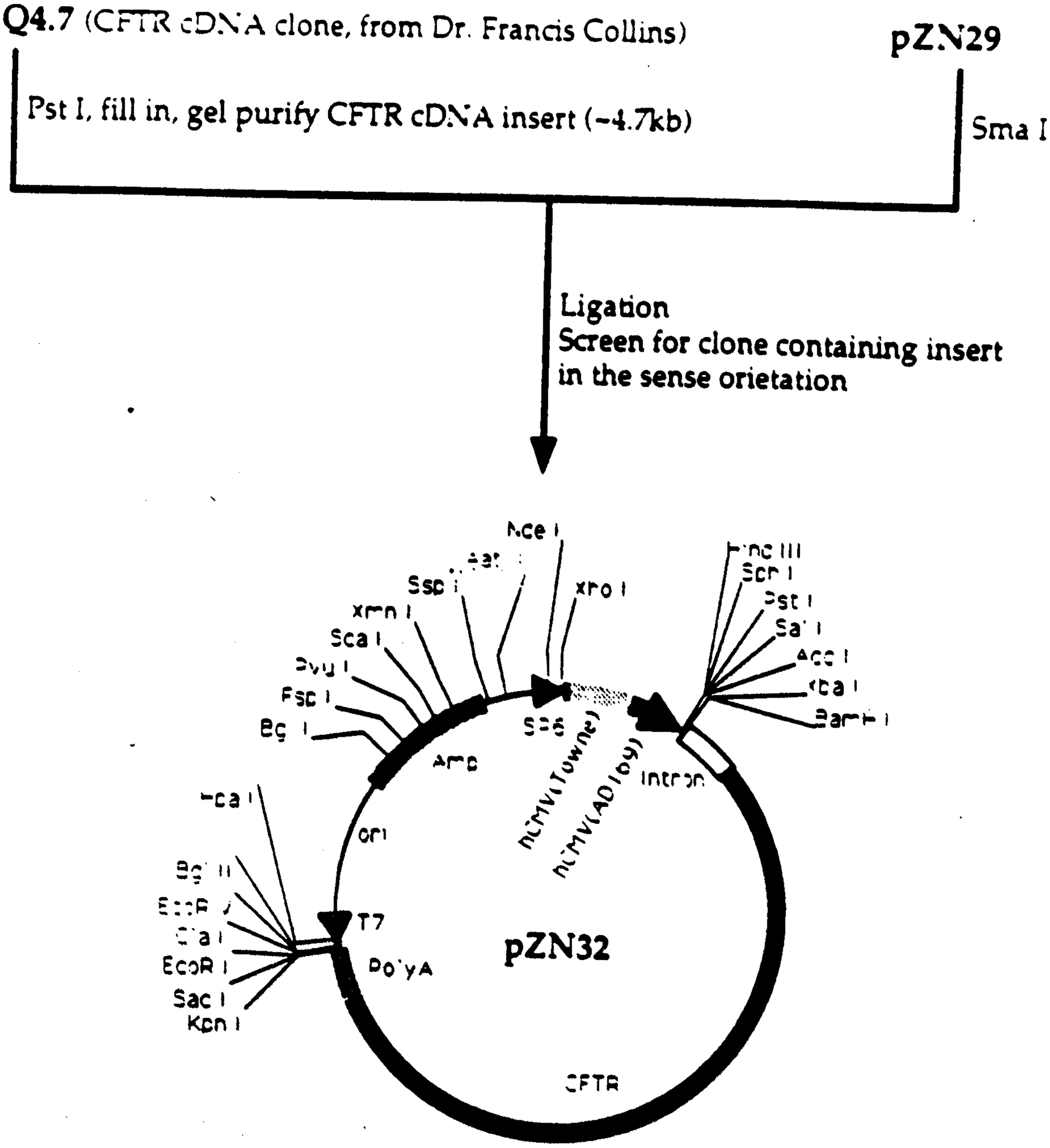
pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)
Pst I, fill in, gel purify CFTR cDNA insert (~4.7kb)
pZN29
Sma I
Ligation
Screen for clone containing insert
in the sense orietation
pZN32
CFTR
Amp
ori
T7
Poly A
SD6
hCMV(Towne)
hCMV(ADIoy)
Intron
Nce I
Xho I
Sca I
Sac I
Kpn I
EcoR I
Bgl II
Hind III
Sal I
Acc I
Xba I
BamH I

FIGURE 5

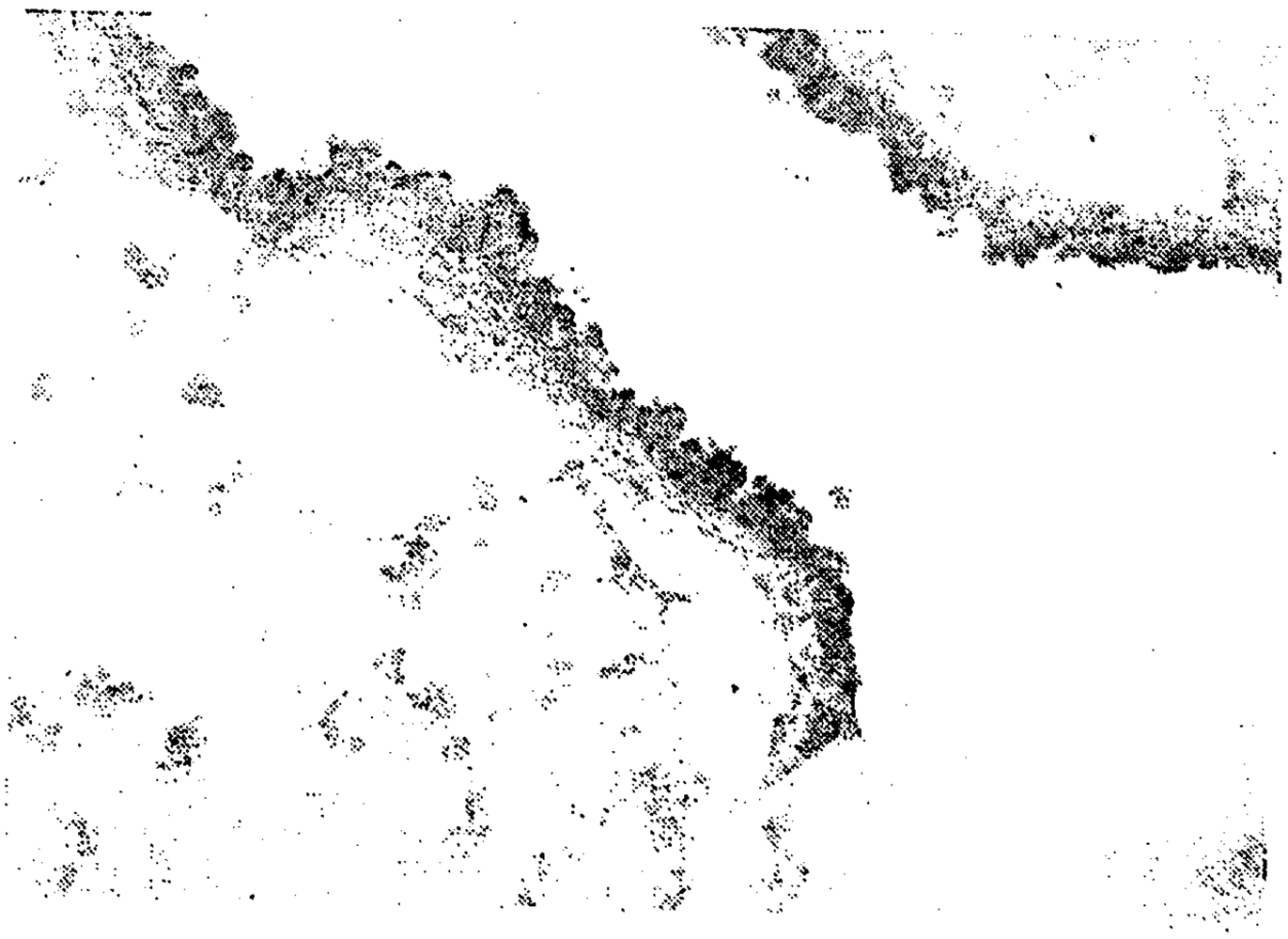

FIGURE 6A

FIGURE 6B

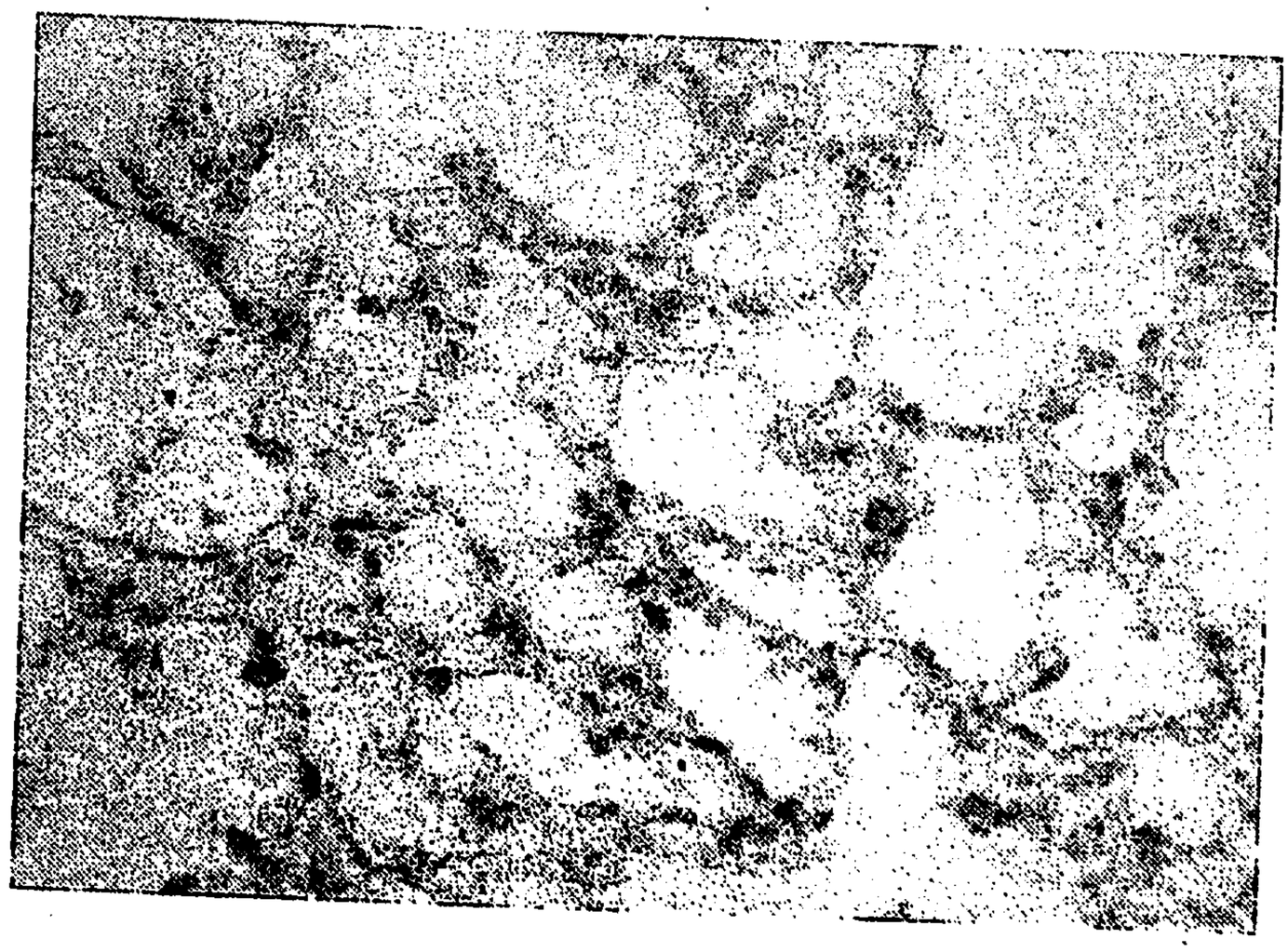

FIGURE 6C

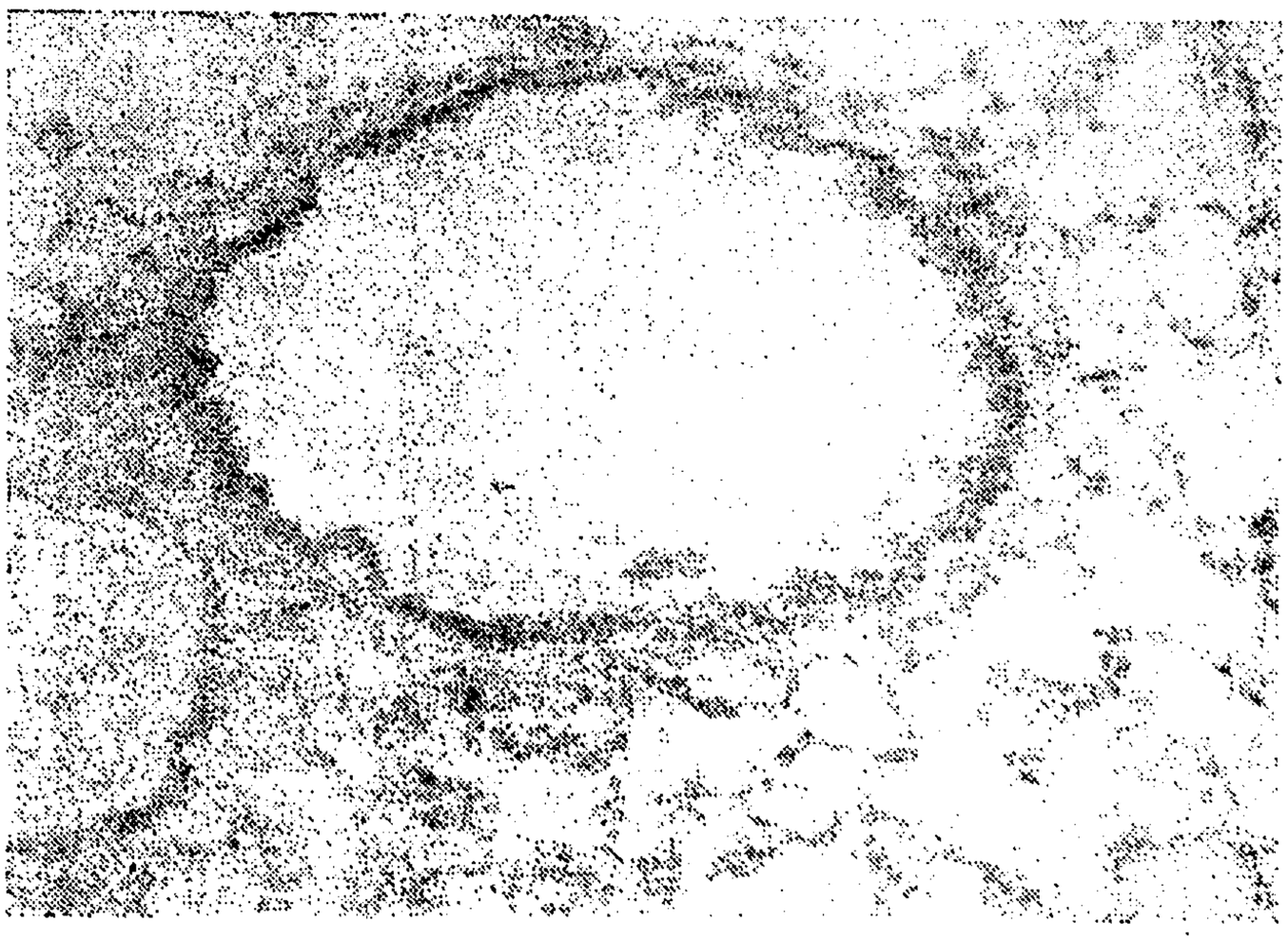

FIGURE 6D

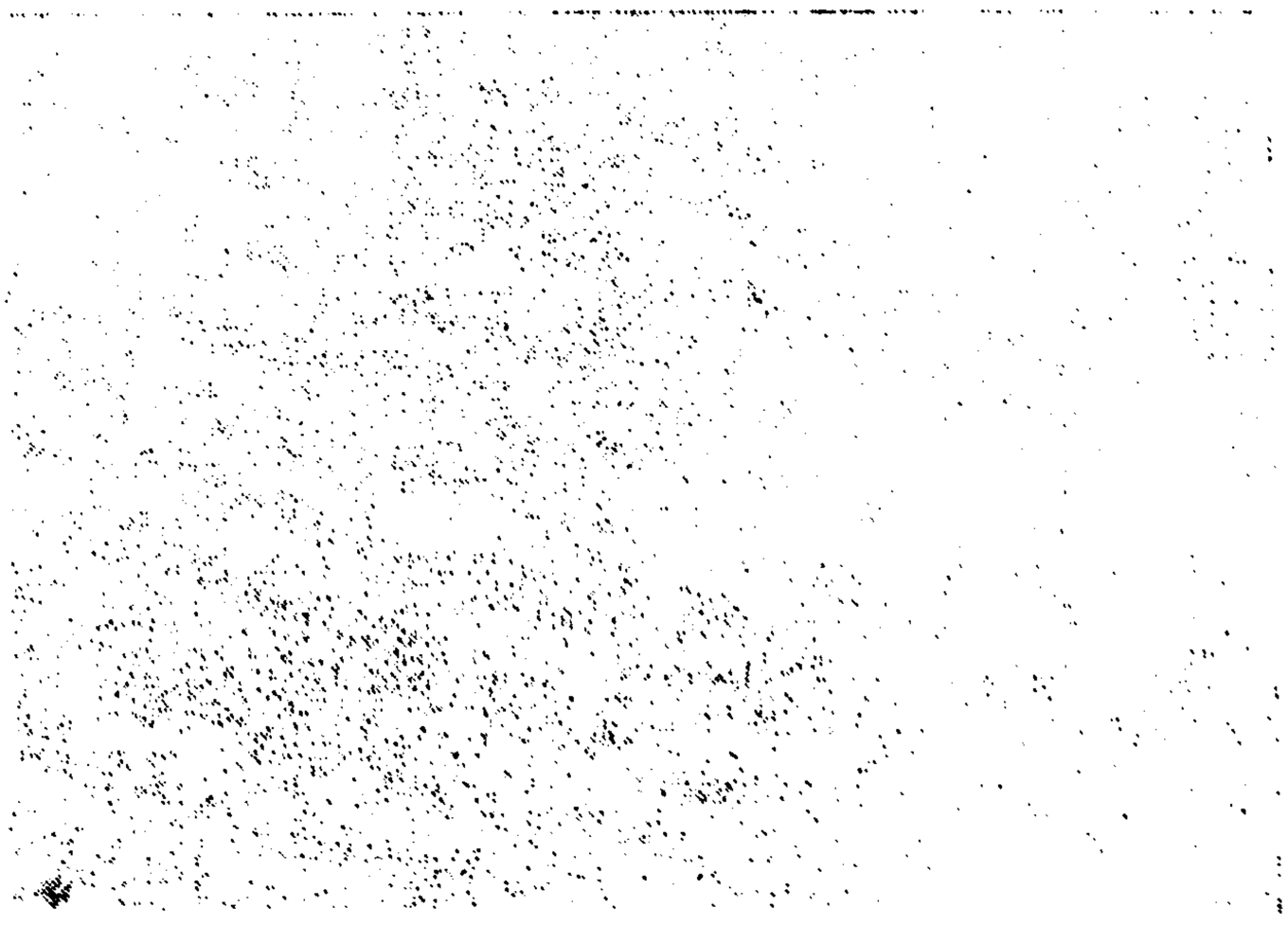

FIGURE 6E

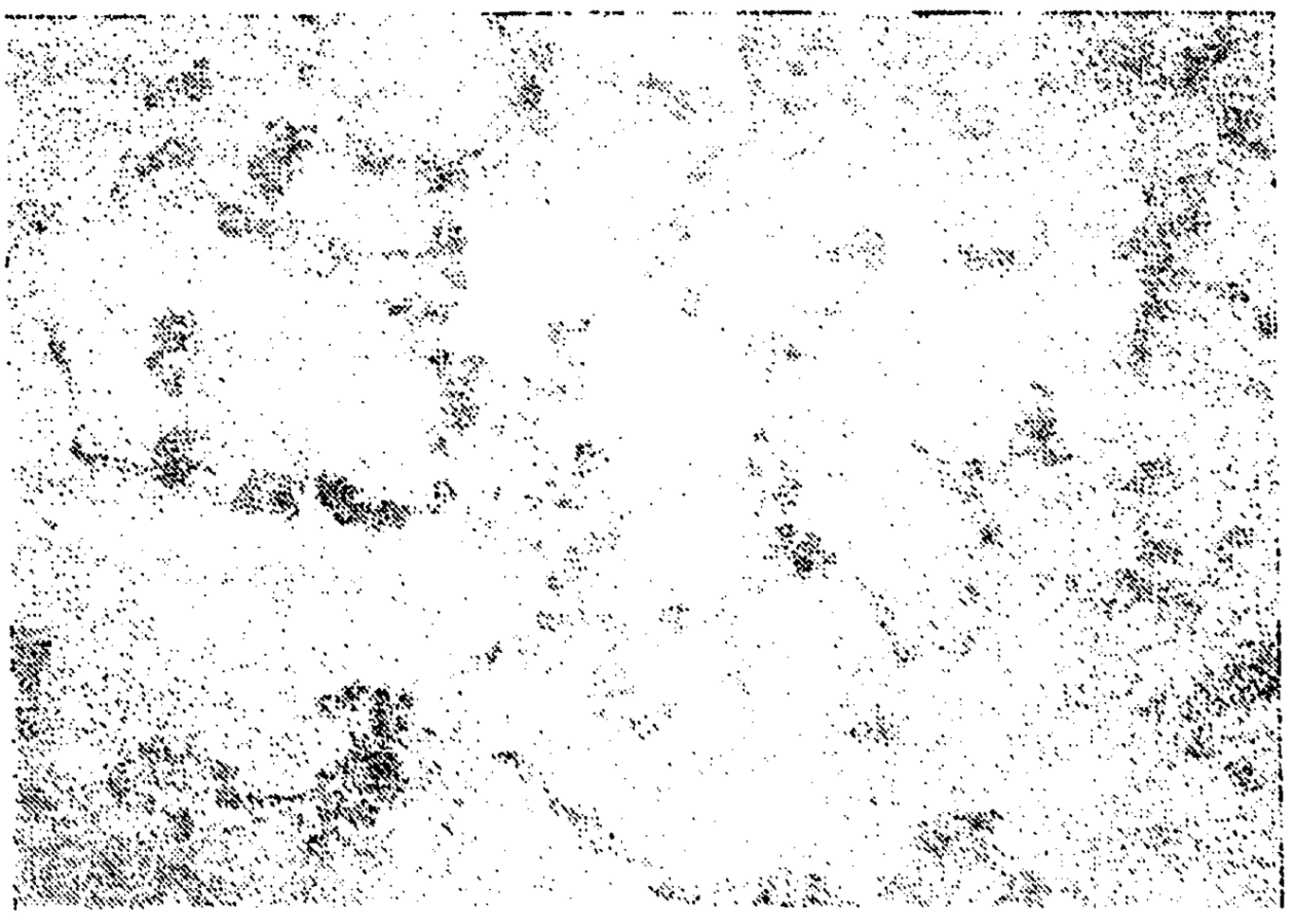

FIGURE 6F

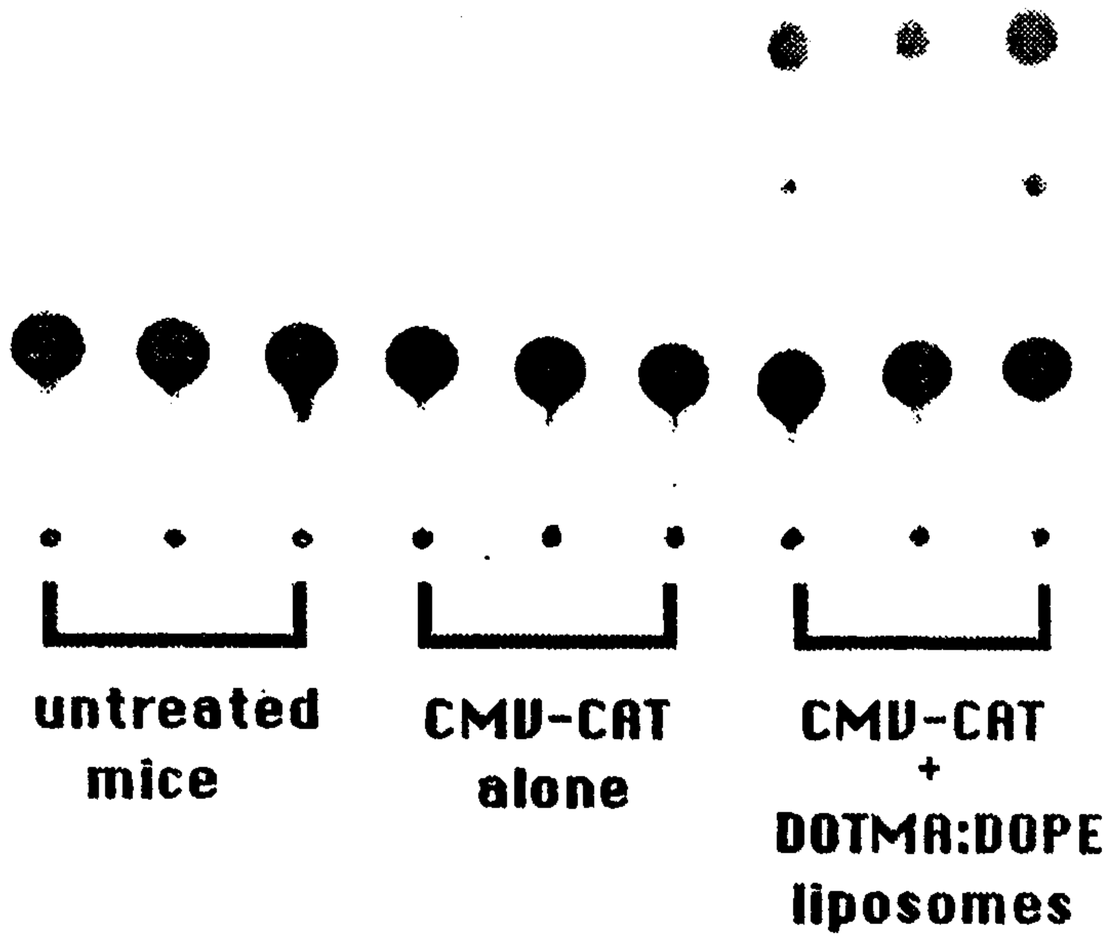

FIGURE 7

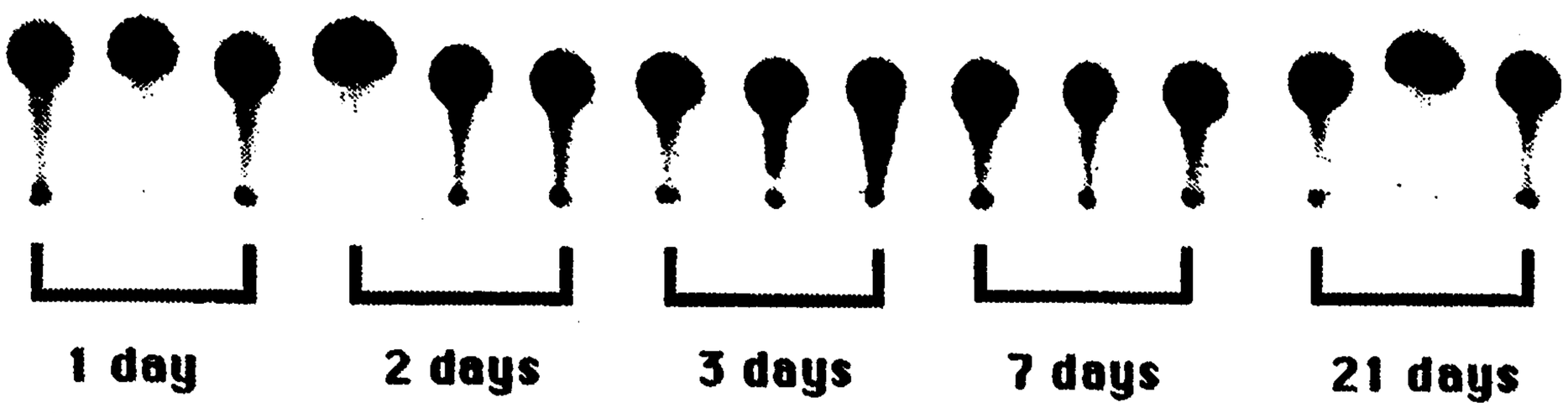

FIGURE 8a

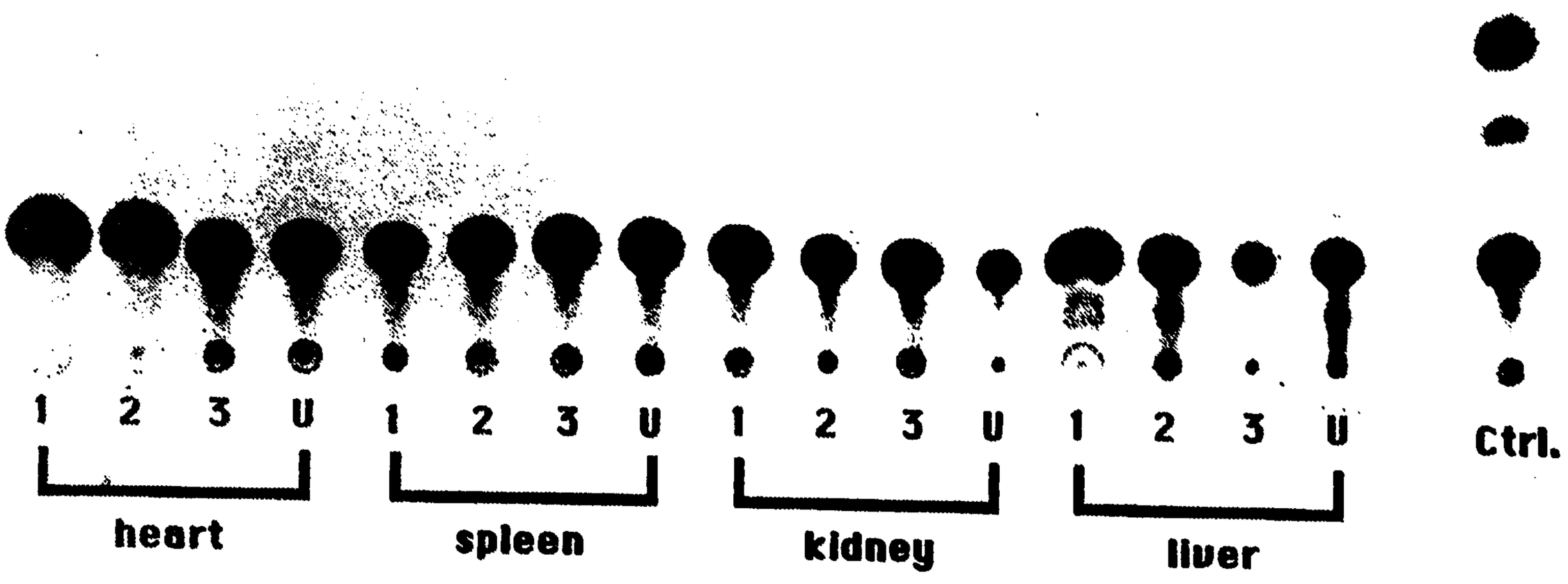

FIGURE 8b

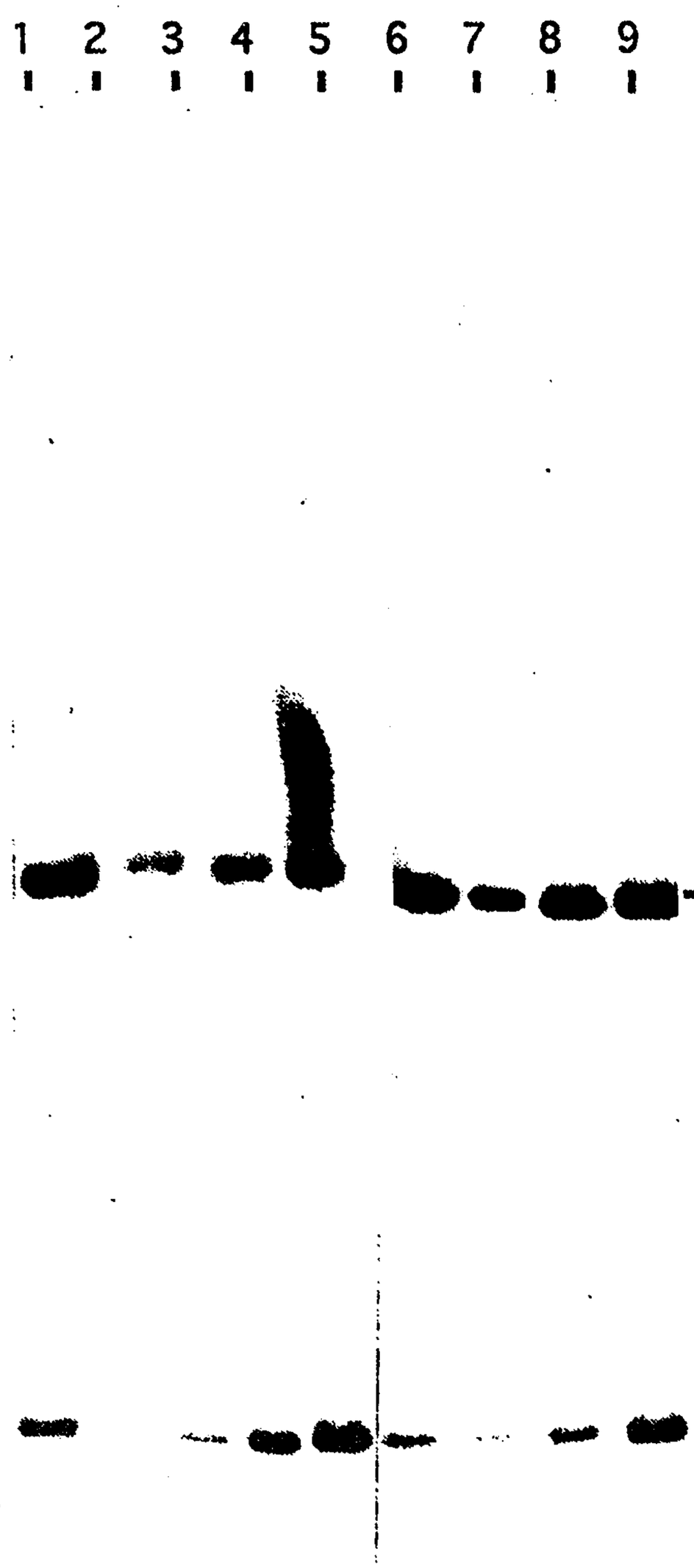

FIGURE 9

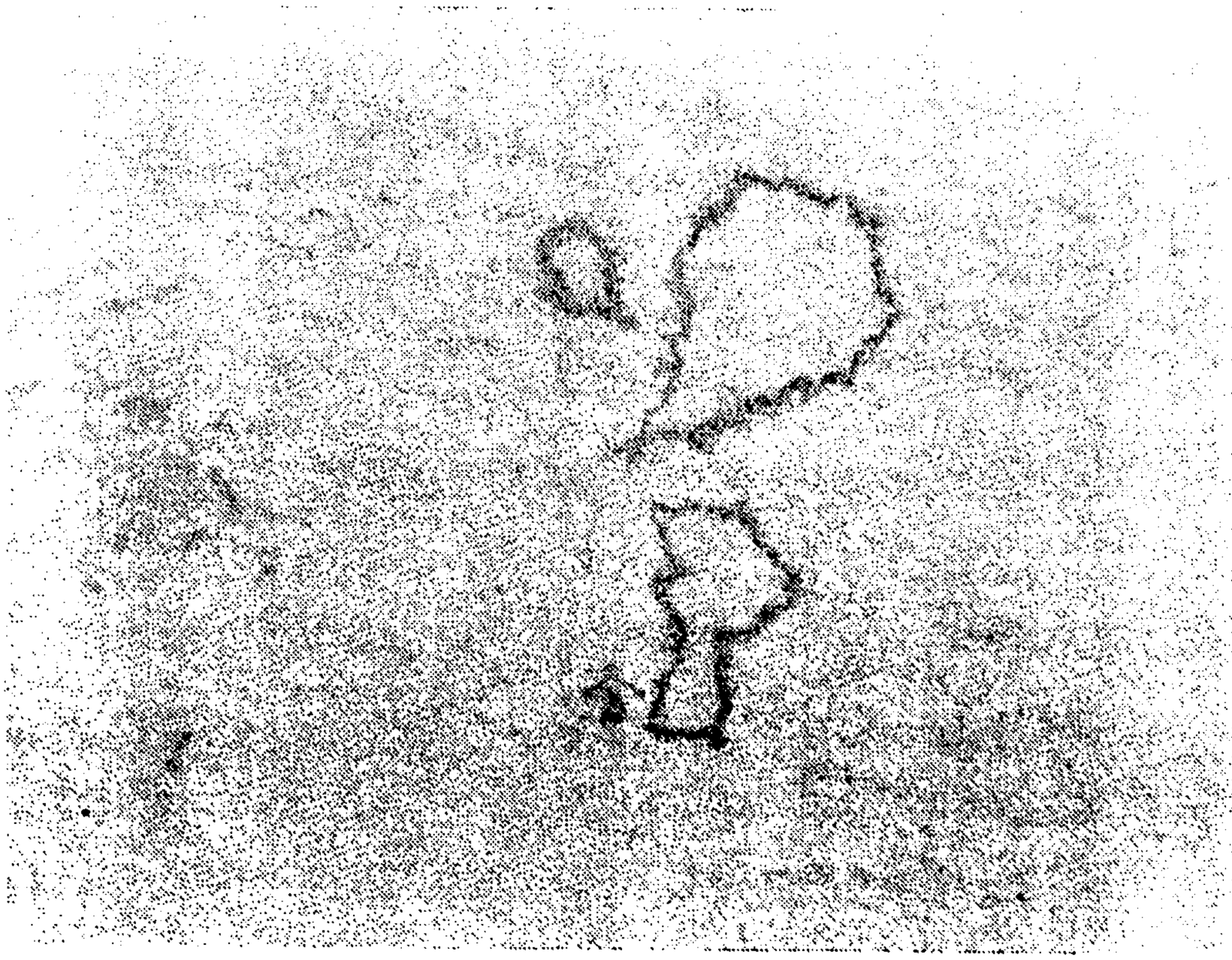

FIGURE 10A

FIGURE 10B

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FIGURE 10C

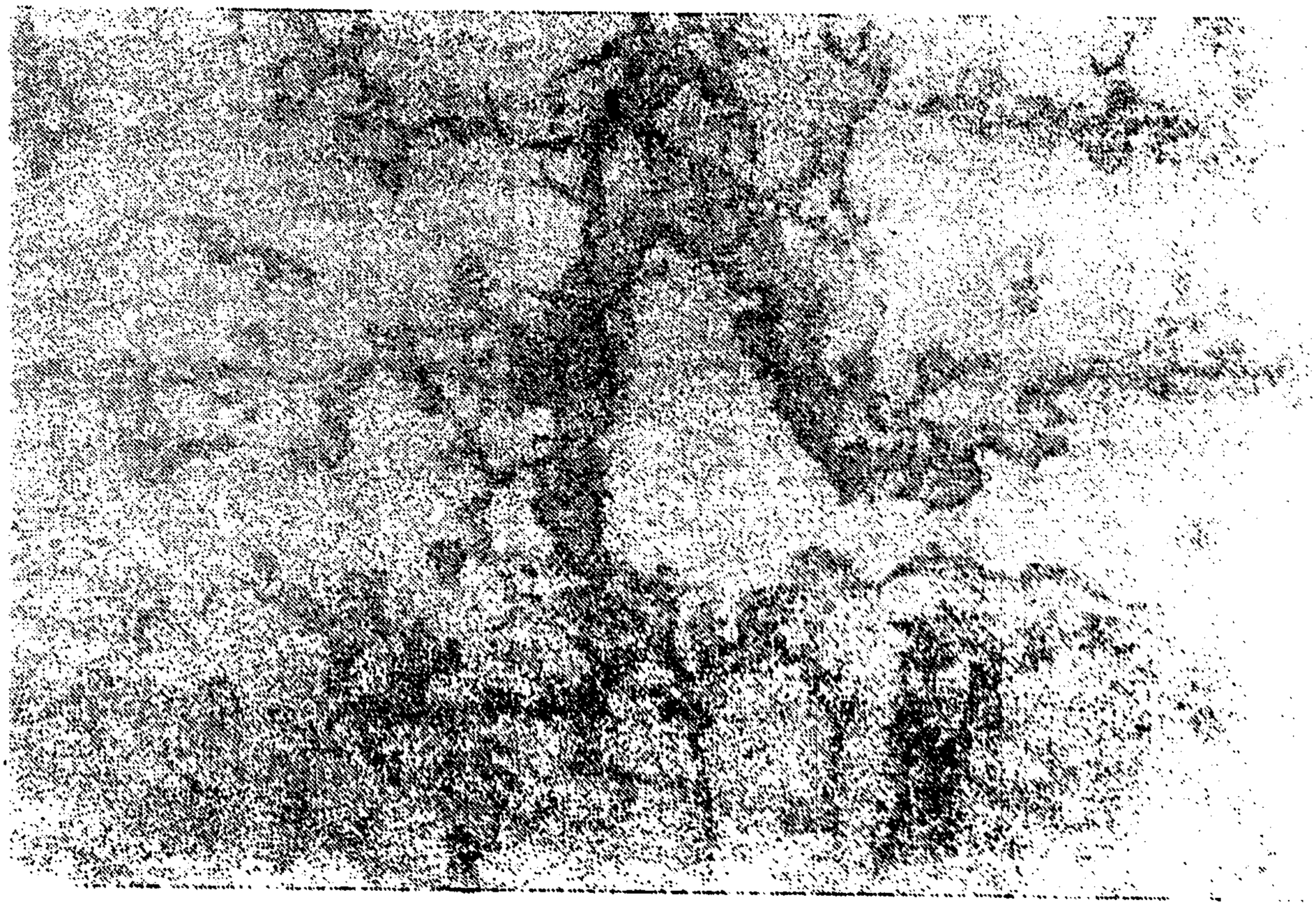

FIGURE 10D

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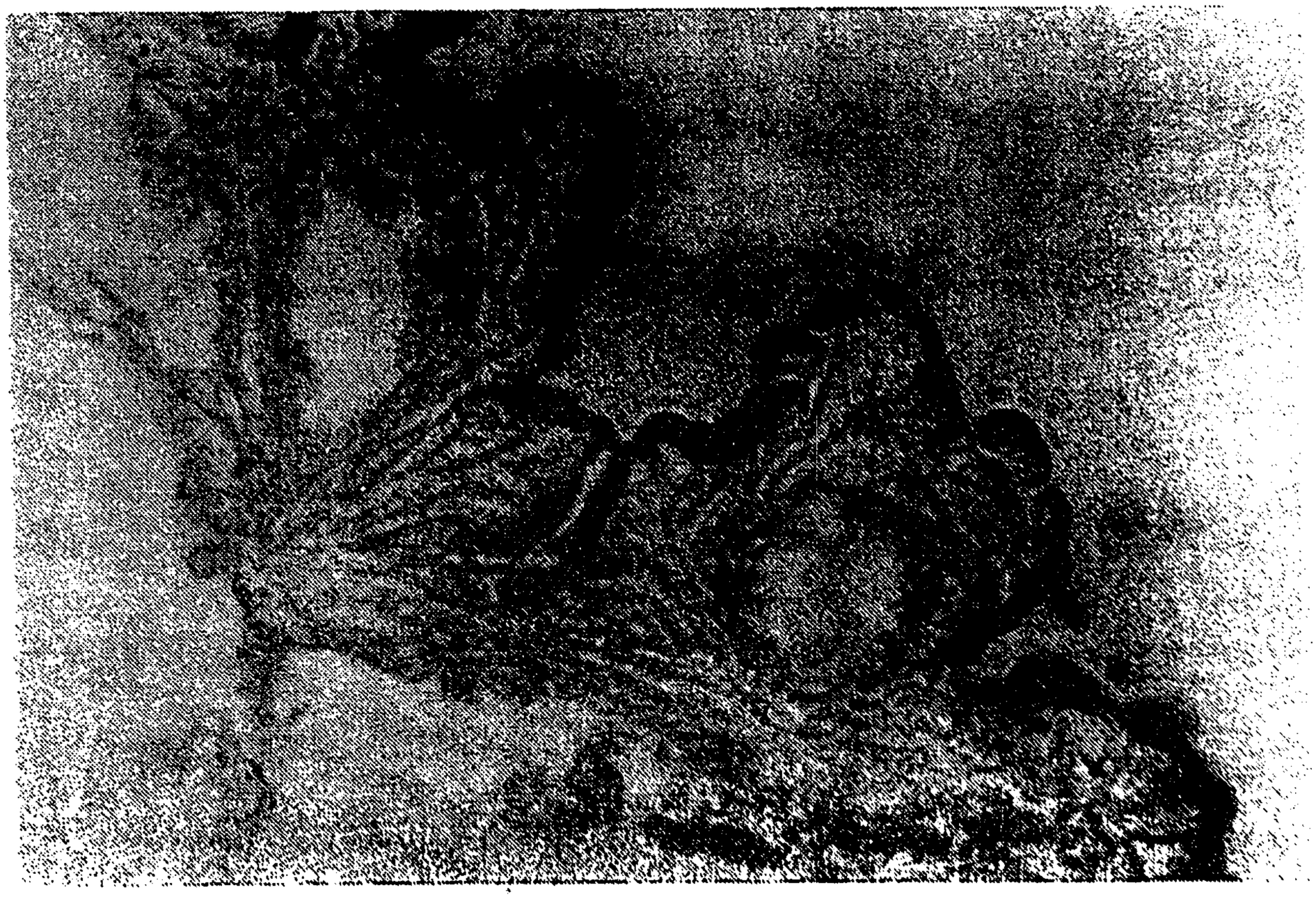


FIGURE 10E

DNA Strider™ 1.. Tuesday, March 3, 1992 7:25:33 PM

HCMV (Towne) -> Full Restriction Map

DNA sequence 616 b.p. ggcgaccgccca ... agtgacgtaagt linear

Positions of Restriction Endonucleases sites (unique sites underlined)

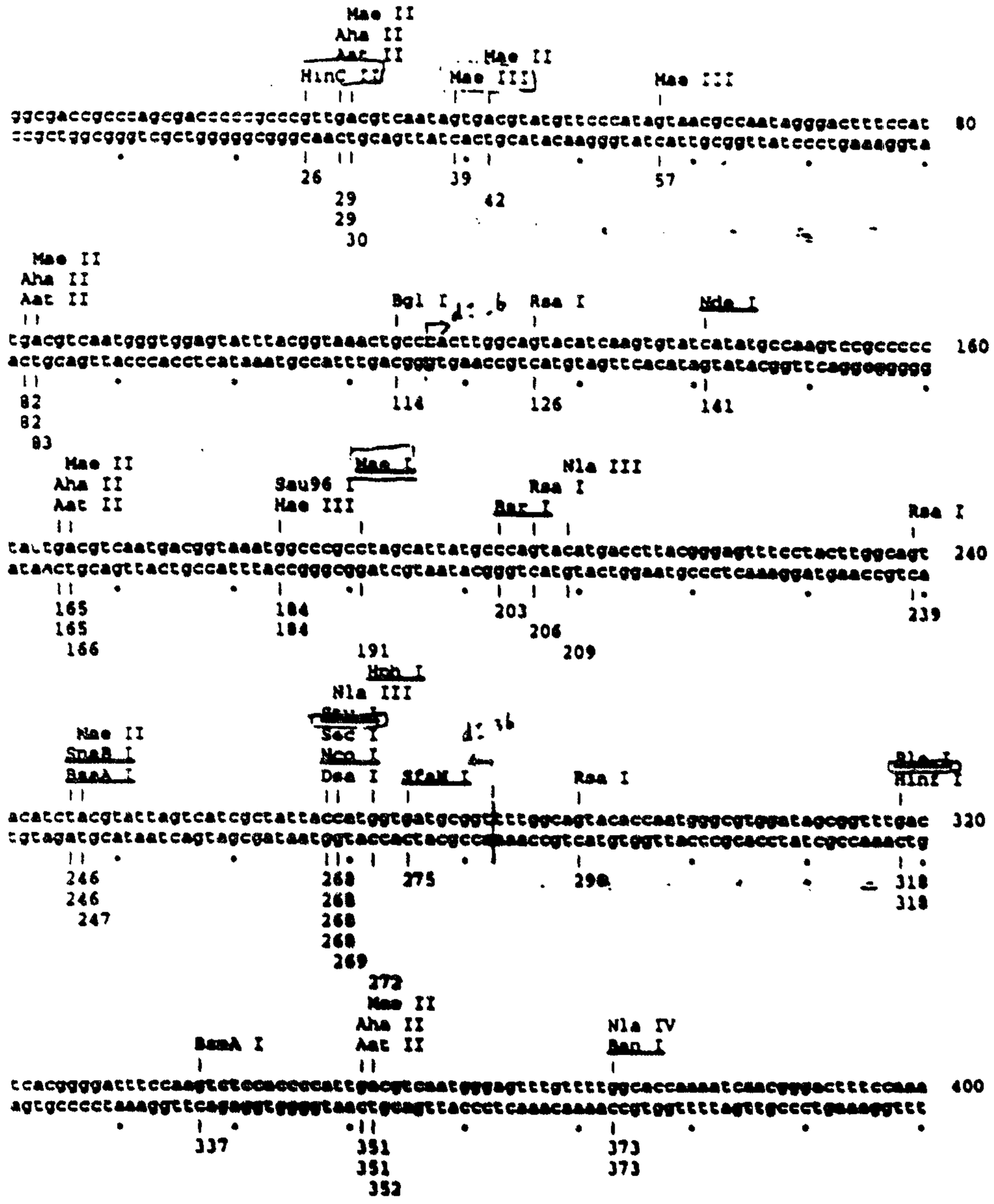
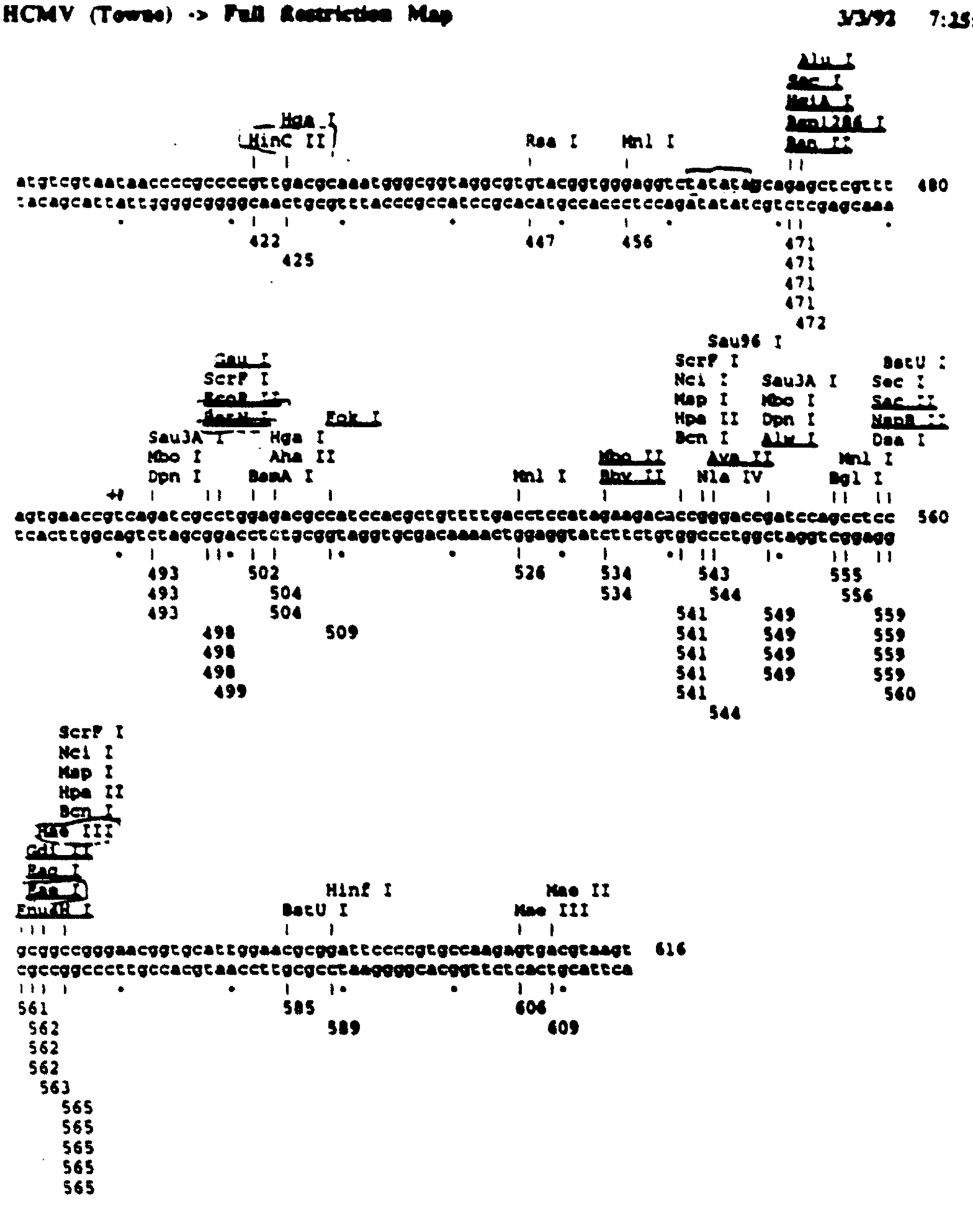


FIGURE 11A(1)



Restriction Endonucleases site usage

Aat II	4	BstN I	1	HinC II	2	Ple I	1
Acc I	-	BstU I	2	HinD III	-	Pml I	-
Afl II	-	BstX I	-	Hinf I	2	PpuM I	-
Afl III	-	BstY I	-	HinP I	-	Pst I	-
Aha II	5	Bsu36 I	-	Hpa I	-	Pvu I	-
Alu I	1	Cfr10 I	-	Hpa II	2	Pvu II	-
Alw I	1	Cla I	-	Hph I	1	Rsa I	5
AlwN I	-	Dde I	-	Kpn I	-	Rsr II	-
Apa I	-	Dpn I	2	Mae I	1	Sac I	1
ApaL I	-	Dra I	-	Mae II	7	Sac II	1
Ase I	-	Dra III	-	Mae III	3	Sal I	-
Asp718	-	Drd I	-	Mbo I	2	Sau3A I	2
Ava I	-	Dsa I	2	Mbo II	1	Sau96 I	2
Ava II	1	Eae I	1	Mlu I	-	Sca I	-
Avr II	-	Eag I	1	Mme I	-	ScrF I	3

FIGURE 11A(2)

HCMV (Towne) -> Pl Restriction Map 3/3/92 7:25:33 PM Page 3

BamH I	-	Ear I	-	Mnl I	3	Sec I	2				
Ban I	1	Eco47 III	-	Mse I	-	SfaN I	1				
Ban II	1	Eco57 I	-	Mse I	-	Sfi I	-				
Bbe I	-	EcoN I	-	Msp I	2	Sma I	-				
Bbv I	-	Eco0109 I	-	Nae I	-	SnaB I	1				
Bbv II	1	EcoR I	-	Nar I	-	Spe I	-				
Bcl I	-	EcoR II	1	Nci I	2	Sph I	-				
Bcn I	2	EcoR V	-	Nco I	1	Spl I	-				
Bgl I	2	Esp I	-	Nde I	1	Sap I	-				
Bgl II	-	Fnu4H I	1	Nhe I	-	Stu I	-				
BseA I	1	Fok I	1	Nla III	2	Sty I	1				
Bsm I	-	Fsp I	-	Nla IV	2	Taq I	-				
BsmA I	2	Gdi II	1	Not I	-	Tth111 I	-				
Bsp1286 I	1	Gsu I	1	Nru I	-	Tth111 II	-				
BspH I	-	Hae I	-	Nsi I	-	Xba I	-				
BspM I	-	Hae II	-	Nsp7524 I	-	Xca I	-				
BspM II	-	Hae III	2	NspB II	1	Xho I	-				
Bsr I	1	Hga I	2	NspH I	-	Xcm I	-				
BssH II	-	HgiA I	1	Pac I	-	Xma I	-				
BstB I	-	HgiE II	-	PaeR7 I	-	Xmn I	-				
BstE II	-	Hha I	-	Pfl1M I	-						

Enzyme	Site	Use	Site position (Fragment length) Fragment order								
Alu I	ag/ct		1	1(471) 1		472(145) 2					
Alw I	ggatc	4/5	1	1(548) 1		549(68) 2					
Ava II	g/gwcc		1	1(543) 1		544(73) 2					
Ban I	g/gyrcc		1	1(372) 1		373(244) 2					
Ban II	grgcy/c		1	1(470) 1		471(146) 2					
Bbv II	gaagac	2/6	1	1(533) 1		534(83) 2					
BseA I	yac/gtr		1	1(245) 2		246(371) 1					
Bsp1286 I	gdgch/c		1	1(470) 1		471(146) 2					
Bsr I	actgg	1/-1	1	1(202) 2		203(414) 1					
BstN I	cc/wgg		1	1(497) 1		498(119) 2					
Eae I	y/ggccr		1	1(561) 1		562(55) 2					
Eag I	c/ggccg		1	1(561) 1		562(55) 2					
EcoR II	/ccwgg		1	1(497) 1		498(119) 2					
Fnu4H I	gc/ngc		1	1(560) 1		561(56) 2					
Fok I	ggatg	9/13	1	1(508) 1		509(108) 2					
Gdi II	yggccg	-5/-1	1	1(561) 1		562(55) 2					
Gsu I	ctggag	16/14	1	1(498) 1		499(118) 2					
HgiA I	gwgcw/c		1	1(470) 1		471(146) 2					
Hph I	ggtga	8/7	1	1(271) 2		272(345) 1					
Hae I	c/tag		1	1(190) 2		191(426) 1					
Mbo II	gaaga	8/7	1	1(533) 1		534(83) 2					
Nco I	c/catgg		1	1(267) 2		268(349) 1					
Nde I	ca/tatg		1	1(140) 2		141(476) 1					
NspB II	cmg/ckg		1	1(558) 1		559(58) 2					
Ple I	gagtc	4/5	1	1(317) 1		318(299) 2					
Sac I	gagct/c		1	1(470) 1		471(146) 2					
Sac II	ccgc/gg		1	1(558) 1		559(58) 2					
SfaN I	gcatc	5/9	1	1(274) 2		275(342) 1					
SnaB I	tac/gta		1	1(245) 2		246(371) 1					
Sty I	c/cwwgg		1	1(267) 2		268(349) 1					
Bcn I	cc s/gg		2	1(540) 1		541(24) 3		565(52) 2			
Bgl I	gccnnnn/nggc		2	1(113) 2		114(441) 1		555(62) 3			
BsmA I	gtctc	1/5	2	1(336) 1		337(165) 2		502(115) 3			
BstU I	cg/cg		2	1(559) 1		560(25) 3		585(32) 2			
Dpn I	ga/tc		2	1(492) 1		493(56) 3		549(68) 2			
Dsa I	c/crygg		2	1(267) 2		268(291) 1		559(58) 3			
Hae III	gg/cc		2	1(183) 2		184(379) 1		563(54) 3			
Hga I	gacgc	5/10	2	1(424) 1		425(79) 3		504(113) 2			
HinC II	gty/rac		2	1(25) 3		26(396) 1		422(195) 2			
Hinf I	g/antc		2	1(317) 1		318(271) 2		589(28) 3			
Hpa II	c/cgg		2	1(540) 1		541(24) 3		565(52) 2			
Mbo I	/gatc		2	1(492) 1		493(56) 3		549(68) 2			
Msp I	c/cgg		2	1(540) 1		541(24) 3		565(52) 2			
Nci I	cc/sgg		2	1(540) 1		541(24) 3		565(52) 2			
Nla III	catg/		2	1(208) 2		209(60) 3		269(348) 1			
Nla IV	ggn/ncc		2	1(372) 1		373(170) 2		543(74) 3			
Sau3A I	/gatc		2	1(492) 1		493(56) 3		549(68) 2			
Sau96 I	g/gncc		2	1(183) 2		184(360) 1		544(73) 3			
Sec I	c/cnngg		2	1(267) 2		268(291) 1		559(58) 3			

FIGURE 11A(3)

HCMV (Towne) -> Pst :estriction Map 3/3/92 7:25:33 PM Page 4

Mae III	/gtnac		3	1(	38) 2	39(	18) 3	57(	549) 1	606(	11) 4				
Mnl I	cctc	7/7	3	1(	455) 1	456(	70) 2	526(	30) 4	556(	61) 3				
ScrF I	cc/ngg		3	1(	497) 1	498(	43) 3	541(	24) 4	565(	52) 2				
Aat II	gacgt/c		4	1(	28) 5	29(	53) 4	82(	83) 3	165(	186) 2				
				351(	266) 1										
Aha II	gr/cgyc		5	1(	28) 6	29(	53) 5	82(	83) 4	165(	186) 1				
				351(	153) 2	504(	113) 3								
Rsa I	gt/ac		5	1(	125) 3	126(	80) 4	206(	33) 6	239(	51) 5				
				290(	157) 2	447(	170) 1								
Mae II	a/cgt		7	1(	29) 6	30(	12) 7	42(	41) 5	83(	83) 3				
				166(	81) 4	247(	105) 2	352(	257) 1	609(	8) 8				

98 sites found

No Sites found for the following Restriction Endonucleases

Acc I	gt/mkac	Dra III	cacnnn/gtg		Nsp7524 I	r/catgy	
Afl II	c/ttaag	Drd I	gacnnnnn/nngtc		NspH I	rcatg/y	
Afl III	a/crygt	Ear I	ctcttc	1/4	Pac I	ttaat/taa	
AlwN I	cagnnn/ctg	Eco47 III	agc/gct		PaeR7 I	c/tcgag	
Apa I	gggcc/c	Eco57 I	ctgaag	16/14	PflM I	ccannnn/ntgg	
ApaL I	g/tgcac	EcoN I	cctnn/nnnagg		Pml I	cac/gtg	
Ase I	at/taat	Eco0109 I	rg/gnccy		PpuM I	rg/gwccy	
Asp718	g/gtacc	EcoR I	g/aattc		Pst I	ctgca/g	
Ava I	c/ycgrg	EcoR V	gat/atc		Pvu I	cgat/cg	
Avr II	c/ctagg	Esp I	gc/tnagc		Pvu II	cag/ctg	
BamH I	g/gatcc	Fsp I	tgc/gca		Rsr II	cg/gwccg	
Bbe I	ggcgc/c	Hae I	wgg/ccw		Sal I	g/tcgac	
Bbv I	gcagc	8/12	Hae II	rgcgc/y	Sca I	agt/act	
Bcl I	t/gatca	HgiE II	accnnnnnnggt		Sfi I	ggccnnnn/nggcc	
Bgl II	a/gatct	Hha I	gcg/c		Sma I	ccc/ggg	
Bsm I	gaatgc	1/-1	HinD III	a/agctt	Spe I	a/ctagt	
BspH I	t/catga	HinP I	g/cgc		Sph I	gcatg/c	
BspM I	acctgc	4/8	Hpa I	gtt/aac	Spl I	c/gtacg	
BspM II	t/ccgga	Kpn I	ggtac/c		Sse I	aat/att	
BssH II	g/cgcgc	Mlu I	a/cgcgt		Stu I	agg/cct	
BstB I	tt/cgaa	Mme I	tccrac	20/18	Taq I	t/cga	
BstE II	g/gtnacc	Mac I	tgg/cca		Tth111 I	gacn/nngtc	
BstX I	ccannnnn/ntgg	Mse I	t/taa		Tth111 II	caarca	11/9
BstY I	r/gatcy	Nae I	gcc/ggc		Xba I	t/ctaga	
Bsu36 I	cc/tnagg	Nar I	gg/cgcc		Xca I	gta/tac	
Cfr10 I	r/ccggy	Nhe I	g/ctagc		Xho I	c/tcgag	
Cla I	at/cgat	Not I	gc/ggccgc		Xcm I	ccannnnn/nnnntgg	
Dde I	c/tnag	Nru I	tcg/cga		Xmn I	gaann/nnttc	
Dra I	ttt/aaa	Nsi I	atgca/t				

FIGURE 11A(4)

```
Socrates

   *** Aligned sequences:
   C1 ( 1f): |>u 1>+++++ ad169hcmv (930 bases)+++++>u 930>|
   C2 ( 1f): |>u 1>+++++ hs5miel (616 bases)+++++>u 616>|

   *** Alignment of first sequence with all others displayed
   *** Key:
       UPPER CASE = aligned non-identical bases
       lower case = unaligned bases
       ---------- = aligned identical bases
       .......... = gap

         ad169hcmv : AATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGC
           hs5miel : ..................................................

         ad169hcmv : TATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGT
           hs5miel : ..................................................
                                                  ↱ CMV-1
         ad169hcmv : CCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACG
           hs5miel : ..................................................

         ad169hcmv : GGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
           hs5miel : ..................................................

         ad169hcmv : CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCC
           hs5miel : .....---.---------G----------G----------G----------

         ad169hcmv : ATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACT
           hs5miel : ----------------------------------------------------------

         ad169hcmv : GCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAAT
           hs5miel : ----------C-----------------------------------------------

         ad169hcmv : GACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACT
           hs5miel : ----------A----------C----G---------------------------------

         ad169hcmv : TGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTAC
           hs5miel : ----------------------------------------------------------

         ad169hcmv : ATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC
           hs5miel : -C--------------------------------------------------------

         ad169hcmv : GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAAC
           hs5miel : ----------------------------------------------------T---

         ad169hcmv : TCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGA
           hs5miel : C-------G--------------------------------------.------

         ad169hcmv : GCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCAT
           hs5miel : ----------------------------------------------------------

         ad169hcmv : AGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATT
           hs5miel : ----------------------------------------------------------

         ad169hcmv : CCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCT
           hs5miel : ----------.....................................

         ad169hcmv : TCTTATGCATGCTATACTGTTTTTGGCTTG
           hs5miel : ...............................
```

FIGURE 11B(1)

```
Socrates                                                              1.

LOCUS       HS5IEE          930 bp ds-DNA              VRL       15-SEP-1989
DEFINITION  Human cytomegalovirus major immediate-early gene, enhancer.
ACCESSION   K03104
KEYWORDS    major immediate-early gene.
SOURCE      HCMV strain AD169.
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1  (bases 1 to 930)
  AUTHORS   Boshart,M., Weber,F., Jahn,G., Dorsch-Haesler,K., Fleckenstein,B.
            and Schaffner,W.
  TITLE     A very strong enhancer is located upstream of an immediate early
            gene of human cytomegalovirus
  JOURNAL   Cell 41, 521-530 (1985)
  STANDARD  full staff_review
REFERENCE   2  (sites)
  AUTHORS   Zhang,X.-Y., Inamdar,N.M., Supakar,P.C., Wu,K., Ehrlich,M. and
            Ehrlich,K.C.
  TITLE     three MDBP sites in the immediate-early enhancer-promoter region of
            human cytomegalovirus
  JOURNAL   Virology 182, 865-869 (1991)
  STANDARD  full staff_review
COMMENT     Draft entry and printed copy of sequence in [1] were kindly
            provided by M.Boshart, 24-OCT-1985.
FEATURES             Location/Qualifiers
     misc_signal     214..620
                     /note="HCMV IE enhancer region"
     mRNA            738..>930
                     /note="HCMV IE mRNA"
BASE COUNT      233 a    228 c    211 g    258 t
ORIGIN      12 bp upstream of BalI site; .750 mu.
        1 aatcaatatt ggccattagc catattattc attggttata tagcataaat caatattggc
       61 tattggccat tgcatacgtt gtatccatat cataatatgt acatttatat tggctcatgt
      121 ccaacattac cgccatgttg acattgatta ttgactagtt attaatagta atcaattacg
      181 gggtcattag ttcatagccc atatatggag ttccgcgtta cataacttac ggtaaatggc
      241 ccgcctggct gaccgcccaa cgacccccgc ccattgacgt caataatgac gtatgttccc
      301 atagtaacgc caatagggac tttccattga cgtcaatggg tggagtattt acggtaaact
      361 gcccacttgg cagtacatca agtgtatcat atgccaagta cgcccccctat tgacgtcaat
      421 gacggtaaat ggcccgcctg gcattatgcc cagtacatga ccttatggga ctttcctact
      481 tggcagtaca tctacgtatt agtcatcgct attaccatgg tgatgcggtt ttggcagtac
      541 atcaatgggc gtggatagcg gtttgactca cggggatttc caagtctcca ccccattgac
      601 gtcaatggga gtttgttttg gcaccaaaat caacgggact ttccaaaatg tcgtaacaac
      661 tccgccccat tgacgcaaat gggcggtagg cgtgtacggt gggaggtcta tataagcaga
      721 gctcgtttag tgaaccgtca gatcgcctgg agacgccatc cacgctgttt tgacctccat
      781 agaagacacc gggaccgatc cagcctccgc ggccgggaac ggtgcattgg aacgcggatt
      841 ccccgtgcca agagtgacgt aagtaccgcc tatagagtct ataggcccac cccttggct
      901 tcttatgcat gctatactgt ttttggcttg
```

1

FIGURE 11B(2)

```
Socrates                                                              3/3
LOCUS       HSSMIE1      616 bp ds-DNA              VRL     15-SEP-1989
DEFINITION  Human cytomegalovirus (Towne) major immediate-early (IE) gene, exon
            1.
ACCESSION   K01484 K01090
KEYWORDS    major immediate-early gene.
SOURCE      Human cytomegalovirus (strain Towne) passed in primary human
            foreskin fibroblasts. DNA [1], clone pXEP22 [2].
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1  (bases 460 to 616)
  AUTHORS   Stenberg,R.M., Thomsen,D.R. and Stinski,M.F.
  TITLE     Structural analysis of the major immediate early gene of human
            cytomegalovirus
  JOURNAL   J. Virol. 49, 190-199 (1984)
  STANDARD  full staff_review
REFERENCE   2  (bases 1 to 490)

Enable your screen-to-printer option; tap RETURN
EuGene Project: CMV report: (GenBank entry information)
LOCUS       HSSMIE1      616 bp ds-DNA              VRL     15-SEP-1989
DEFINITION  Human cytomegalovirus (Towne) major immediate-early (IE) gene, exon
            1.
ACCESSION   K01484 K01090
KEYWORDS    major immediate-early gene.
SOURCE      Human cytomegalovirus (strain Towne) passed in primary human
            foreskin fibroblasts. DNA [1], clone pXEP22 [2].
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1  (bases 460 to 616)
  AUTHORS   Stenberg,R.M., Thomsen,D.R. and Stinski,M.F.
  TITLE     Structural analysis of the major immediate early gene of human
            cytomegalovirus
  JOURNAL   J. Virol. 49, 190-199 (1984)
  STANDARD  full staff_review
REFERENCE   2  (bases 1 to 490)
  AUTHORS   Thomsen,D.R., Stenberg,R.M., Goins,W.F. and Stinski,M.F.
  TITLE     Promoter-regulatory region of the major immediate early gene of
            human cytomegalovirus
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 81, 659-663 (1984)
  STANDARD  full staff_review
COMMENT     IE region 1 gene is also known as the major IE gene.
FEATURES             Location/Qualifiers
     prim_transcript 490..>616
                     /note="major IE mRNA"
     intron          611..>616
                     /note="major IE mRNA intron A"
BASE COUNT      144 a    165 c    162 g    145 t
ORIGIN      28 bp upstream of HincII site; 0.752 map units.
        1 ggcgaccgcc cagcgacccc cgcccgttga cgtcaatagt gacgtatgtt cccatagtaa
       61 cgccaatagg gactttccat tgacgtcaat gggtgggagta tttacggtaa actgcccact
      121 tggcagtaca tcaagtgtat catatgccaa gtccgccccc tattgacgtc aatgacggta
      181 aatggcccgc ctggcattat gcccagtaca tgaccttacg ggagtttcct acttggcagt
      241 acatctacgt attagtcatc gctattacca tggtgatgcg gttttggcag tacaccaatg
      301 ggcgtggata gcggtttgac tcacggggat ttccaagtct ccaccccatt gacgtcaatg
      361 ggagtttgtt ttggcaccaa aatcaacggg actttccaaa atgtcgtaat aaccccgccc
      421 cgttgacgca aatgggcggt aggcgtgtac ggtgggaggt ctatataagc gagctcgttt
      481 agtgaaccgt cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca
      541 ccgggaccga tccagcctcc gcggccggga acggtgcatt ggaacgcgga ttccccgtgc
      601 caagagtgac gtaagt
```

1

FIGURE 11B(3)

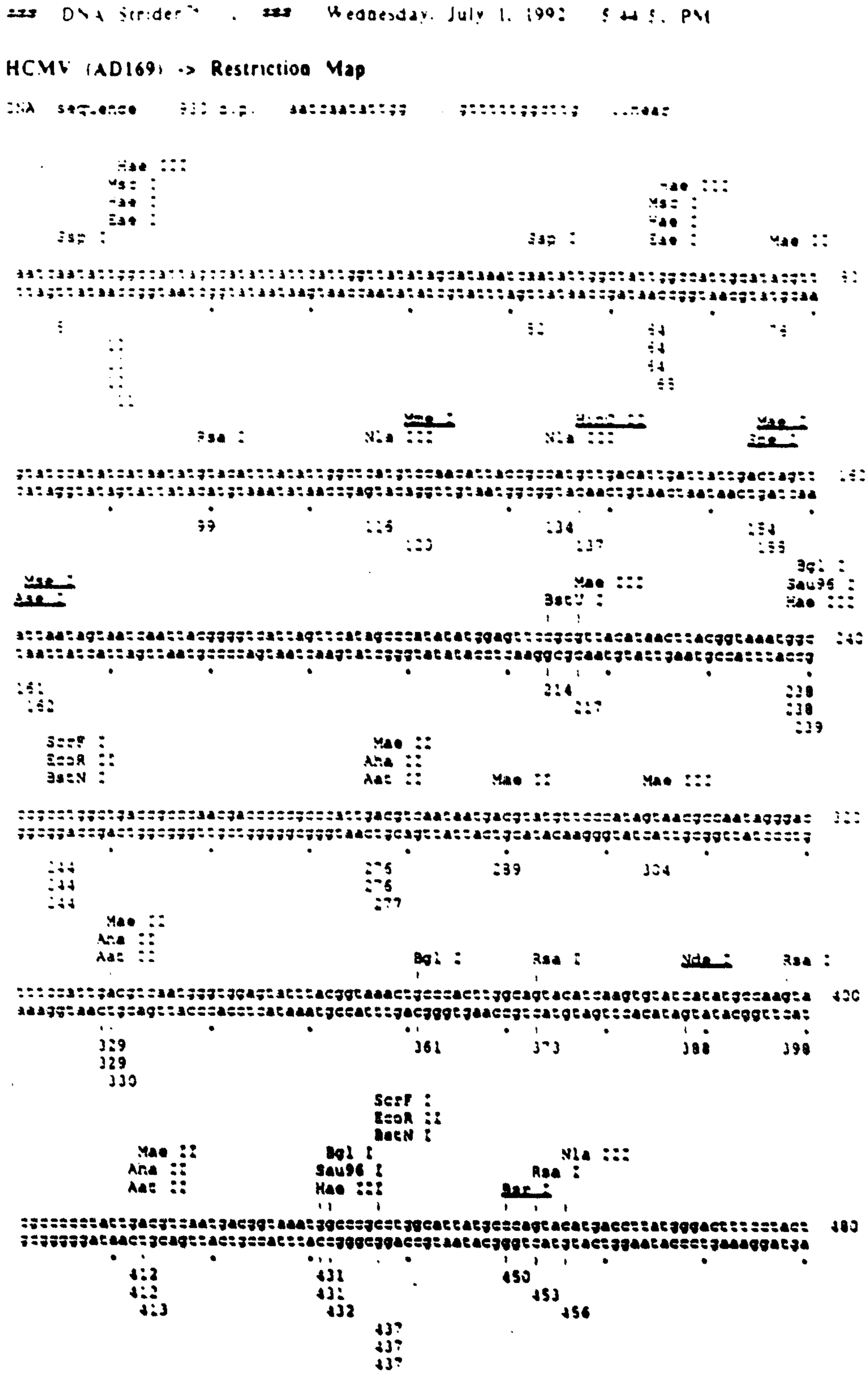

DNA Strider
Wednesday, July 1, 1992
HCMV (AD169) -> Restriction Map
DNA sequence
Hae III
Msc I
Nae I
Bae I
Bsp I
Mae II
Pst I
Nla III
Mae I
Eae I
Bgl I
Sau96 I
BstU I
ScrF I
EcoR II
BstN I
Aha II
Aat II
Rsa I
Nde I
Bar I

FIGURE 11C(1)

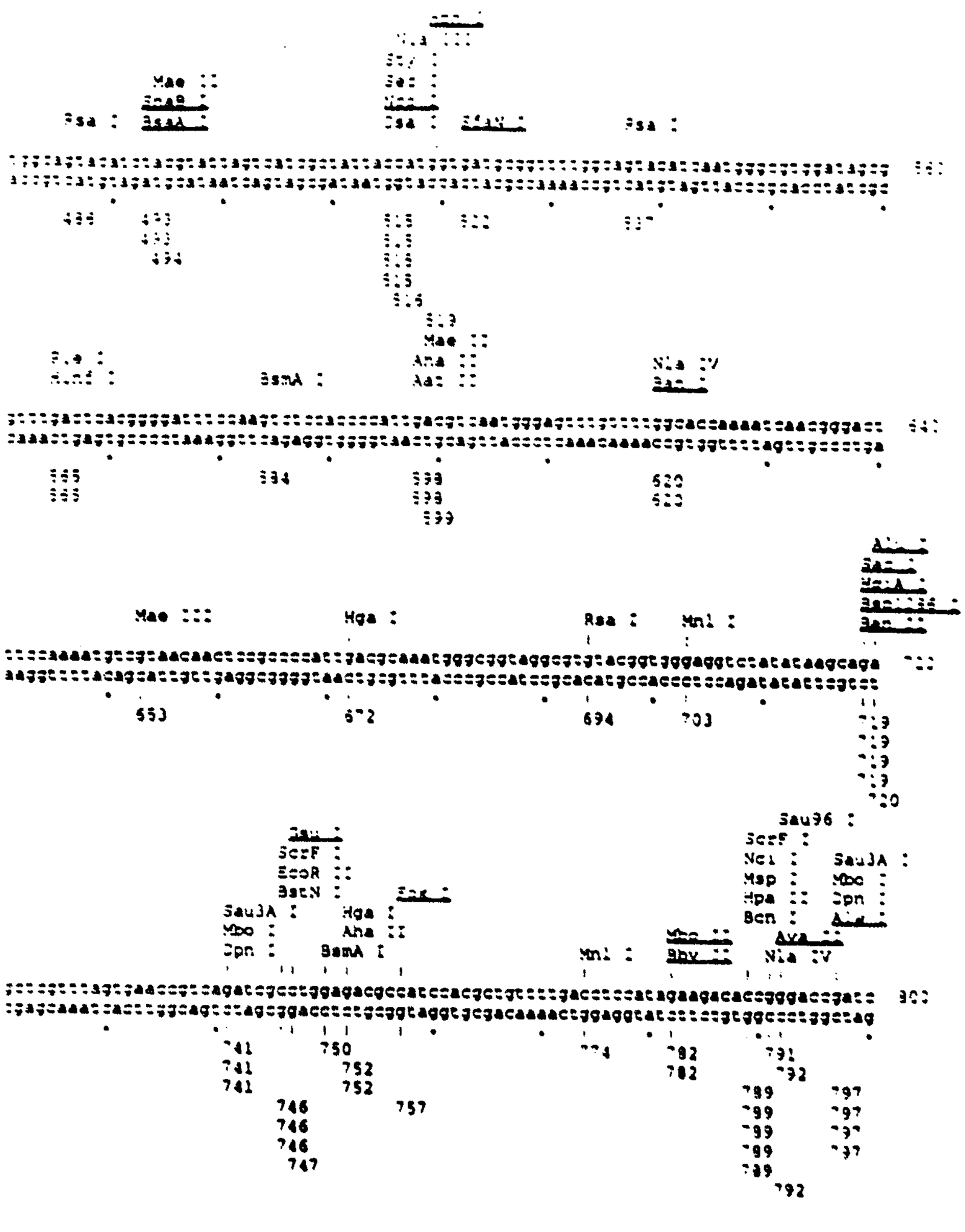

Friction Map
Mae II
BsaA I
Bsa I
Bsa I
Bsa I
Mae II
Ama II
Aat II
Nla IV
Bst I
BsmA I
Mae III
Hga I
Rsa I
Mnl I
Bst II
Sau96 I
ScrF I
Nci I
Sau3A I
Mbo I
Dpn I
Hga I
Aha II
BamA I
Mnl I
Bbv II
Nla IV

FIGURE 11C(2)

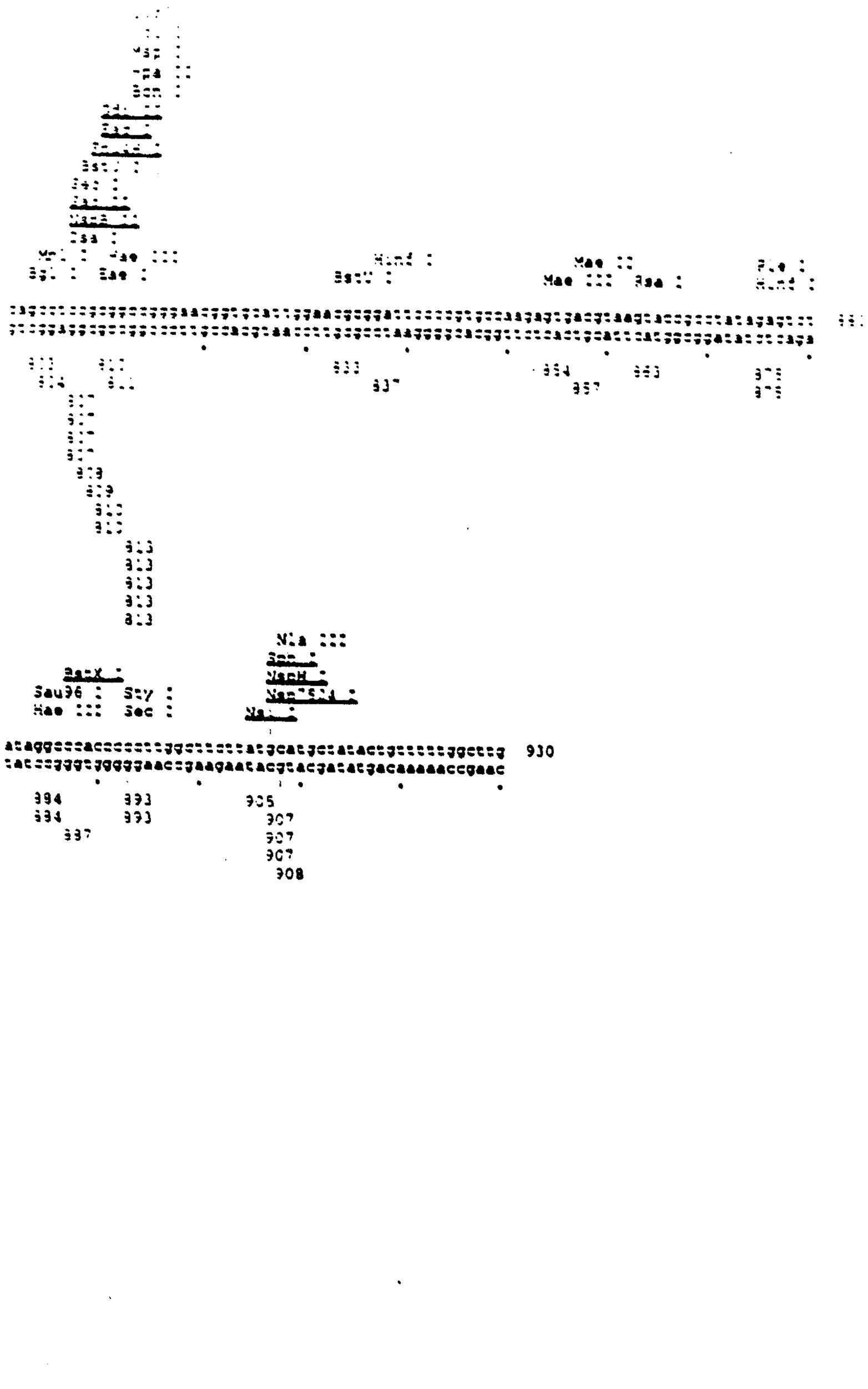

HCMV AD169 -> Restriction Map
Bam
Sac
Mae III
Hind
Mae II
Eco
Bgl
Eae
Bst
Mae III
Bsa
Mlu
Sau96
Sty
Sac
Nla III
Bam
NarH
Nar GI A
Nar
BamX
Sau96
Sty
Sac
Hae III
Nla
934
933
935
937
907
908

FIGURE 11C(3)