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(54) Title: VP2-MODIFIED RAAV VECTORS AND USES THEREOF

(57) **Abrégé/Abstract:**

Disclosed are improved VP2-modified recombinant adeno-associated viral (rAAV) vectors, expression systems, and rAAV virions that are fully virulent, yet lack functional VP2 protein expression. Also disclosed are pharmaceutical compositions, virus particles, host cells, and pharmaceutical formulations that comprise these modified vectors useful in the expression of therapeutic proteins, polypeptides, peptides, antisense oligonucleotides and/or ribozymes in the cells and tissues of selected mammals, including, for example, human tissues and host cells.



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(54) Title: VP2-MODIFIED RAAV VECTORS AND USES THEREOF

(57) Abstract: Disclosed are improved VP2-modified recombinant adeno-associated viral (rAAV) vectors, expression systems, and rAAV virions that are fully virulent, yet lack functional VP2 protein expression. Also disclosed are pharmaceutical compositions, virus particles, host cells, and pharmaceutical formulations that comprise these modified vectors useful in the expression of therapeutic proteins, polypeptides, peptides, antisense oligonucleotides and/or ribozymes in the cells and tissues of selected mammals, including, for example, human tissues and host cells.

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VP2 - MODIFIED RAAV VECTORS AND USES THEREOF

1. BACKGROUND OF THE INVENTION

The present application claims priority to United States Provisional Application Serial No. 60/377,315, filed May 1, 2002, and Intl. Pat. Appl. Ser. No. PCT/US03/13583, filed May 1, 2003, the entire contents of each of which is specifically incorporated herein by reference in its entirety. The United States government has certain rights in the present invention pursuant to grant numbers P50 HL59412, PO1 HL51811 and T32 AI 7110 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and virology, and in particular, to the development of gene delivery vehicles. The invention provides VP2-modified recombinant adeno-associated virus (rAAV) vectors that, while deleted for VP2, are still fully virulent. Methods are provided for preparing and using these modified rAAV-based vector constructs in a variety of viral-based gene therapies, and in particular, in the treatment, amelioration, and/or prevention of human diseases.

1.2 DESCRIPTION OF RELATED ART

Major advances in the field of gene therapy have been achieved by using viruses to deliver therapeutic genetic material. The adeno-associated virus (AAV) has attracted considerable attention as a highly effective viral vector for gene therapy due to its low immunogenicity and ability to effectively transduce non-dividing cells. AAV has been shown to infect a variety of cell and tissue types by using heparin sulfate proteoglycan (HSPG) as its primary cellular receptor. The natural tropism of AAV for the abundantly expressed HSPG presents a challenge to specifically targeting particular cell populations. For safety and targeting considerations it is highly desirable to have a vector that cannot infect its natural host cell types.

2. SUMMARY OF THE INVENTION

The present invention overcomes these and other limitations inherent in the prior art by providing new rAAV-based genetic constructs that encode one or more mammalian therapeutic polypeptides for the prevention, treatment, and/or amelioration of various

disorders resulting from a deficiency in one or more of such polypeptides. In particular, the invention provides AAV-based genetic constructs encoding one or more mammalian therapeutic proteins, polypeptides, peptides, antisense oligonucleotides, and ribozymes, as well as variants, and/or active fragments thereof, for use in the treatment and prophylaxis of a variety of conditions and mammalian diseases and disorders.

Current AAV2 targeting strategies involve inserting DNA sequences that code for specific receptor ligands within the capsid open reading frame of the pIM45 plasmid. While this approach has identified surface positions capable of tolerating peptide insertions, there are certain limitations. Because the three capsid proteins share the same open reading frame and stop codon, the amino acid sequence of the major capsid protein, VP3, and any peptide ligands inserted in this region of the open reading frame, are contained within the 2 larger and significantly less abundant capsid proteins, VP1 and VP2.

In order to target peptide ligands to a specific capsid protein, the inventors have investigated an alternative method for the production of recombinant AAV2 vectors. By mutating the capsid proteins' start codons the inventors have generated pIM45 plasmids that only express one capsid protein: pIM45-VP1, pIM45-VP2 (acg/atg), and pIM45-VP3. Such plasmids can be complemented with plasmids that express the remaining 2 capsid proteins (pIM45-VP2,3, pIM45-VP1,3, and pIM45-VP1,2, respectively) in order to produce viable recombinant AAV2 vectors. Interestingly, the plasmid, pIM45-VP1,3 is also capable of producing infectious virions in the absence of VP2 expression. Expression of the capsid proteins in this manner allows for the genetic modification of a specific capsid protein across its entire sequence. As a result, more control of the position and number of expressed peptide insertions is obtained in producing recombinant AAV2 vectors. This system allows for the production of novel targeted recombinant AAV2 vectors containing significantly larger peptide insertions in an individual capsid protein without disruption of the remaining capsid structure.

In one embodiment, the invention concerns rAAV vectors that comprise a nucleic acid segment modified to express functional VP1 and VP3 capsid proteins substantially in the absence of functional VP2 protein. Surprisingly, the inventors have shown that such a vector can produce an infectious virion in the absence of exogenous VP2 protein.

The lack or substantial absence of functional VP2 protein may be the result of at least a first mutation in the capsid gene sequence region that comprises the VP2 start codon, or alternatively in the VP2 start codon itself. An exemplary vector described herein is pIM45-VP1,3.

In another embodiment, the invention concerns rAAV vectors that comprise a nucleic acid segment modified to express functional VP1 and VP2 capsid proteins substantially in the absence of functional VP3 protein. Although such vector cannot produce an infectious virion in the absence of exogenous VP3 protein, if a second helper vector that encodes a functional VP3 protein is employed to coinfect cells with this vector, infectious virions can be obtained.

The lack or substantial absence of functional VP3 protein may be the result of at least a first mutation in the capsid gene sequence region that comprises the VP3 start codon, or alternatively in the VP3 start codon itself. An exemplary vector described herein is pIM45-VP1,2.

In a third embodiment, the invention concerns rAAV vectors that comprise a nucleic acid segment modified to express functional VP2 and VP3 capsid proteins substantially in the absence of functional VP1 protein. Although such vector cannot produce an infectious virion in the absence of exogenous VP1 protein, if a second helper vector that encodes a functional VP1 protein is employed to coinfect cells with this vector, infectious virions can be obtained.

The lack or substantial absence of functional VP1 protein may be the result of at least a first mutation in the capsid gene sequence region that comprises the VP1 start codon, or alternatively in the VP1 start codon itself. An exemplary vector described herein is pIM45-VP2,3.

A yet further embodiment of the invention is an expression vector that expresses an rAAV capsid protein selected from the group consisting of VP1, VP2, and VP3, each in the absence of substantially any other rAAV protein, such as the other capsid proteins or helper functions.

This expression vector may comprise, for example, a mutation at position 1 of the cap gene, a mutation at position 138 of the cap gene, or a mutation at position 203 of the cap gene. Exemplary such vectors provided herein are pIM45-VP1, pIM45-VP2, or pIM45-VP3, which produce substantially a single VP1, VP2, or VP3 protein, respectively.

Another embodiment of the invention is an expression vector that expresses: (a) rAAV capsid proteins VP1 and VP2 in the absence of substantial amounts of VP3 protein; (b) rAAV capsid proteins VP1 and VP3 in the absence of substantial amounts of VP2 protein; or (c) rAAV capsid proteins VP2 and VP3 in the absence of substantial amounts of VP1 protein.

Such vectors typically comprise: (a) at least one mutation in the start codon of the VP1 protein and at least one mutation in the start codon of the VP2 protein; (b) at least one mutation in the start codon of the VP1 protein and at least one mutation in the start codon of the VP3 protein; or (c) at least one mutation in the start codon of the VP2 protein and at least one mutation in the start codon of the VP3 capsid protein.

For example, the vector may comprise: (a) at least one mutation at position 1 and at least one mutation at position 138 of the cap gene, (b) at least one mutation at position 1 and at least one mutation at position 203 of the cap gene; or (c) at least one mutation at position 138 and at least one mutation at position 203 of the cap gene. Vectors pIM45-VP1,2; pIM45-VP1,3; and pIM45-VP2,3 described herein, are representative examples of each of such vectors, respectively.

The invention also provides in an important embodiment, an rAAV expression system substantially lacking in expression of VP2 protein. This VP2-free system comprises: (a) at least a first rAAV vector comprising at least a first heterologous nucleic acid segment inserted into the capsid sequence region, with the segment encoding at least a first heterologous peptide; and (b) at least a second expression vector that expresses functional VP1 and VP3 capsid proteins in the absence of substantial quantities of VP2 protein, or at least a second and a third expression vector that separately express functional VP1 and VP3 capsid proteins, each of these second and third plasmids expressing a single VP1 or VP3 protein, both in the absence of substantial amounts of VP2 protein.

For example, the system will preferably comprise, consist essentially of, or consist of, at least a first rAAV vector that substantially lacks VP2 expression. Such expression systems will give rise to infectious virions, so long as the helper plasmids provide sufficient exogenous VP1 and VP3 protein to permit the rAAV vector to form the capsid.

In one embodiment, when it is desirable to "target" particular cells, cell surfaces, or cell surface ligands or receptors, it may be desirable to alter the sequence of the capsid gene through the addition of one or more relatively short nucleic acid segments that encode at least 1 or more targeting peptides that, when these heterologous peptides are expressed on the surface of an rAAV virion comprising the vector, the peptide sequence contained within the altered capsid protein will permit the selective targeting of the rAAV virions comprising them to one or more specific types of cells, cell surfaces, or cell surface receptors when the particles are used to transfect a plurality, population, or subpopulation of selected host cells. The inventors contemplate that the exploitation of such targeting peptide sequences, when expressed on the surface of the rAAV virions as contained within the capsid proteins, may be

critical in localizing, enhancing, improving, or increasing the specificity of the rAAV virions for a particular cell type, or may even be useful in permitting transduction of cells or cell types that previously were not appropriate host cells for AAV infection. Such methods could be particularly desirable in altering the native affinity of one or more of the various known serotypes of AAV to one or more host cells not previously capable of efficient transfection by one or more particular serotypes. For example, by appropriate insertion of one or more peptide epitopes, ligands, or recognition sequences, an rAAV serotype 1 vector may be able to efficiently transfect a cell line not readily transfected by wild-type rAAV1 vectors. Likewise, an rAAV serotype 2 vector may be sufficiently modified by addition of appropriate targeting ligands to effectively transfect one or more cell lines, cells types, tissues, or organs, not previously capable of efficient transfection using the unmodified wild-type rAAV2 vector.

As such, preferred embodiments include those VP2-free rAAV expression systems, wherein at least a first peptide inserted into one or more of the capsid protein sequences, permits the rAAV virion to transfect a specific organ tissue, or host cell, with a higher efficiency than an unmodified rAAV vector.

The VP2-free rAAV expression systems of the invention may utilize any rAAV vector, including those of serotypes 1, 2, 3, 4, 5, or 6, and may employ at least two helper plasmids such as pIM45-VP1, pIM45-VP2, or pIM45-VP3, as the second and third expression vectors required in the system to provide exogenous VP1, VP2, and/or VP3 as may be required for efficient virion formation by the rAAV vectors. When only a second helper plasmid is desired, a single vector may be employed such as, for example, pIM45-VP1,3. Alternatively, so long as at least VP1 and VP3 are provided to the system, either on a single plasmid, each on separate plasmids, or by exogenous supplementation of one or both of the purified protein(s) themselves, a fully functional, fully virulent rAAV virion may be reconstituted from the disclosed expression system, either in the presence of functional VP2 protein, or alternatively, substantially in the absence of any endogenously- or exogenously-provided VP2 protein.

When the use of such vectors is contemplated for introduction of one or more exogenous proteins, polypeptides, peptides, ribozymes, and/or antisense oligonucleotides, to a particular cell transfected with the vector, one may employ the rAAV vectors or the VP2-free rAAV expression systems disclosed herein by genetically modifying the vectors to further comprise at least a first exogenous polynucleotide operably positioned downstream and under the control of at least a first heterologous promoter that expresses the

polynucleotide in a cell comprising the vector to produce the encoded peptide, protein, polypeptide, ribozyme, or antisense oligonucleotide. Such constructs may employ heterologous promoters that are constitutive, inducible, or even cell-specific promoters. Exemplary such promoters include, but are not limited to, a CMV promoter, a β -actin promoter, a hybrid CMV promoter, a hybrid β -actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

The vectors or expression systems may also further comprise a second nucleic acid segment that comprises, consists essentially of, or consists of, one or more enhancers, regulatory elements, transcriptional elements, to alter or effect transcription of the heterologous gene cloned in the rAAV vectors. For example, the rAAV vectors of the present invention may further comprise a second nucleic acid segment that comprises, consists essentially of, or consists of, at least a first CMV enhancer, a synthetic enhancer, or a cell- or tissue-specific enhancer. The second nucleic acid segment may also further comprise, consist essentially of, or consist of one or more intron sequences, post-transcriptional regulatory elements, or such like. The vectors and expression systems of the invention may also optionally further comprise a third nucleic acid segment that comprises, consists essentially of, or consists of, one or more polylinker or multiple cloning regions to facilitate insertion of selected genetic elements, polynucleotides, and the like into the vectors and expression constructs at convenient restriction sites.

In other aspects, the invention concerns methods for altering, reducing, or eliminating, the binding of particular rAAV vectors for particular ligands. In an illustrative embodiment, the invention provides rAAV vectors that have altered affinity for heparin, heparin sulfate, and heparin sulfate proteoglycan. This vector comprises at least a first mutation in the capsid gene, wherein the mutation substantially reduces or eliminates the affinity of a viral particle comprising the vector for binding to heparin, heparin sulfate, or heparin sulfate proteoglycan. Preferably, these rAAV vectors comprise one or more Arginine to Alanine mutations, and particularly one or more Arginine to Alanine mutations at position 585 or position 588 of the capsid polypeptide sequence. In rAAV vectors comprising either a single R585A or R588A mutation, or a double mutant comprising both the R585A and the R588A mutations, affinity for heparin sulfate binding by the vector was eliminated. Such vectors are therefore important when one wishes to design improved rAAV vectors that comprise particular capsid protein mutations that either have increased or reduced affinity for one or more particular ligands.

In all aspects of the invention, the exogenous polynucleotides that are comprised within one or more of the improved rAAV vectors disclosed herein will be of mammalian origin, with polynucleotides of human, primate, murine, porcine, bovine, ovine, feline, canine, equine, epine, caprine, or lupine origin being particularly preferred.

As described above, the exogenous polynucleotide will preferably encode one or more proteins, polypeptides, peptides, ribozymes, or antisense polynucleotides, oligonucleotides, PNA molecules, or a combination of two or more of these therapeutic agents. In fact, the exogenous polynucleotide may encode two or more such molecules, or a plurality of such molecules as may be desired. When combinational gene therapies are desired, two or more different molecules may be produced from a single rAAV expression system, or alternatively, a selected host cell may be transfected with two or more unique rAAV expression systems, each of which may comprise a distinct polynucleotide.

In other embodiment, the invention also concerns the disclosed rAAV vectors comprised within an infectious adeno-associated viral particle or virion, or pluralities thereof, which may also be further comprised within one or more pharmaceutical vehicles, formulated for administration to a mammal such as a human for therapeutic, and/or prophylactic gene therapy regimens. Such vectors, virus particles, virions, and pluralities thereof may also be provided in excipient formulations that are acceptable for veterinary administration to selected livestock, exotic or domesticated animals, pets, and the like.

The invention also concerns host cells that comprise at least one of the disclosed rAAV vectors or expression systems. Such host cells are particularly mammalian host cells, with human host cells being particularly highly preferred, and may be either isolated, in cell or tissue culture, or even within the body of the animal itself.

In certain embodiments, the creation of non-human host cells, or isolated human host cells that comprise one or more of the disclosed AAV vectors is also contemplated to be useful for a variety of diagnostic, and laboratory protocols, including, for example, means for the production of large-scale quantities of the rAAV vectors described herein. Such virus production methods are particularly desirable to obtain the often high-titer viral stocks required by many gene therapy protocols.

Compositions comprising one or more of the disclosed rAAV vectors, expression systems, infectious AAV particles, or host cells also form part of the present invention, and particularly those compositions that further comprise at least a first pharmaceutically-acceptable excipient for use in the manufacture of medicaments and methods involving therapeutic administration of such rAAV vectors. Such pharmaceutical compositions may

optionally further comprise liposomes, a lipid, a lipid complex; or the rAAV vectors may be comprised within a microsphere or a nanoparticle. Pharmaceutical formulations suitable for intramuscular, intravenous, or direct injection into an organ or tissue or a plurality of cells or tissues of a human or other mammal are particularly preferred.

Other aspects of the invention concern recombinant adeno-associated virus virion particles, compositions, and host cells that comprise one or more of the AAV vectors disclosed herein, such as for example pharmaceutical formulations of the vectors intended for administration to a mammal through suitable means, such as, by intramuscular, intravenous, or direct injection to cells, tissues, or organs of a selected mammal. Typically, such compositions may be formulated with pharmaceutically-acceptable excipients as described hereinbelow, and may comprise one or more liposomes, lipids, lipid complexes, microspheres or nanoparticle formulations to facilitate administration to the selected organs, tissues, and cells for which therapy is desired.

Kits comprising one or more of the disclosed vectors, virions, viral particles, transformed host cells or pharmaceutical compositions comprising such; and (ii) instructions for using the kit in a therapeutic, diagnostic, or clinical embodiment also represent preferred aspects of the present disclosure. Such kits may further comprise one or more reagents, restriction enzymes, peptides, therapeutics, pharmaceutical compounds, or means for delivery of the compositions to host cells, or to an animal, such as syringes, injectables, and the like. Such kits may be therapeutic kits for treating, preventing, or ameliorating the symptoms of particular diseases, and will typically comprise one or more of the modified AAV vector constructs, expression systems, virion particles, or therapeutic compositions described herein, and instructions for using the kit. Such kits may also be used in large-scale production methodologies to produce large quantities of the viral vectors.

Another important aspect of the present invention concerns methods of use of the disclosed vectors, virions, expression systems, compositions, and host cells described herein in the preparation of medicaments for preventing, treating or ameliorating the symptoms of various diseases, dysfunctions, or deficiencies in an animal, such as a vertebrate mammal. Such methods generally involve administration to a mammal, or human in need thereof, one or more of the disclosed vectors, virions, viral particles, host cells, compositions, or pluralities thereof, in an amount and for a time sufficient to prevent, treat, or lessen the symptoms of such a disease, dysfunction, or deficiency in the affected animal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions,

or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms.

3. BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

FIG. 1A, FIG. 1B and FIG. 1C show generation of plasmids that express two capsid proteins through missense mutation of individual capsid protein start codons. **FIG. 1A** shows mutations required to eliminate VP1 and VP2 expression. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsids following transfection of plasmids, pIM45(lane1); pIM45-VP2,3 (lane2); pIM45-VP1,3 (lane3); and pIM45-M203L (lane 4). Note, lane 4 is the initial attempt to produce plasmid that expresses only VP1 and VP2. Further mutations are required. **FIG. 1B** shows mutations required to eliminate VP3 expression. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsid following transfection of pIM45 (lane1); pIM45-M203L (lane2); pIM45-M203,211L (lane3); pIM45-M203,211,235L (lane4). Note, pIM45-M203,211,235L is designated pIM45-VP1,2. **FIG. 1C** shows alternative mutation used to eliminate VP3 expression while maximizing expression of VP2 protein. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsid proteins following transfection of pIM45 (lane1) and pIM45-VP1,2A (lane3) in which the start codon for VP2 protein is changed from ACG to ATG.

FIG. 2 shows generation of plasmids that express a single capsid protein. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsid proteins following transfection of pIM45 (lane1); pIM45-VP1 (lane2); pIM45-VP2 (lane3) pIM45-VP2A (lane4); pIM45-VP3 (lane5).

FIG. 3A and FIG. 3B show production and purification of rAAV2-like particles that lack expression of specific capsid proteins. **FIG. 3A** shows analysis of effects of missense mutations required to eliminate VP3 expression. Left panel shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from pIM45 (lane1); pIM45-M203L (lane2); pIM45-M211L (lane3); pIM45-M235L (lane4), pIM45-M203,211,235 (lane 5). Right panel shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with

DNaseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe. FIG. 3B shows analysis of effects of eliminating a single capsid on the production and purification of virus particles. Left panel shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from pIM45 (lane1); pIM45-VP1,2 (lane2); pIM45-VP1,3 (lane3); and pIM45-VP2,3 (lane4). Right panel shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with DNaseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe.

FIG. 4 shows complementation capsid plasmid groups employed to produce viable rAAV2 particle preparations. Group VP0 is a control group consisting of pIM45 and pIM45-VP0 (all capsid expression eliminated). Group VP1 is group consisting of pIM45-VP1 and pIM45-VP2,3 in which expression of VP1 is isolated. Group VP2/VP2A is group consisting of pIM45-VP2 or pIM45-VP2A and pIM45-VP1,3 in which expression of VP2 is isolated, and in case of pIM45-VP2A, VP2 expression is maximized. Group VP3 is group consisting of pIM45-VP3 and pIM45-VP1,2 in which expression of VP3 is isolated. Isolation of specific capsid proteins allows genetic modification of the isolated capsid without further modifying remaining capsids. Alternatively, genetic modification of two capsids can be accomplish without further modification of remaining capsid. These groups are cotransfected with pXX6 (Ad helper functions) and pTR-UF5 (terminal repeats flanking expression cassette with CMV promoter driving expression of GFP) to produce rAAV vectors.

FIG. 5A and **FIG. 5B** show production and purification of rAAV2-like particles from complementation groups described in FIG. 4. FIG. 5A, right panel, shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from Group VP0(lane1); Group VP1 (lane2); Group VP2 (lane3); Group VP2A (lane4); and Group VP3 (lane5). Note, lane 4 shows production of particle stock with increased level of VP2 protein in resultant particles composed of all three capsid proteins. FIG. 5A, Right panel shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with DNaseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe. FIG. 5B, left panel, shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified

particle stocks from transfection of pIM45-VP2A and pIM45-VP3 showing production of rAAV2-like particles composed of VP2 and VP3 with increased VP2 levels relative to VP3. FIG. 5B, right panel, shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixanol step gradient were with incubated with DNaseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe.

FIG. 6A, FIG. 6B and FIG. 6C depict production of rAAV2-like particles with large peptide insertions in VP1 and VP2 capsid proteins. FIG. 6A shows production scheme for insertion of large peptides in VP1 and VP2 (top) involves insertion of peptide immediately after amino acid 138 in a plasmid that expresses only VP1 and VP2 (pIM45-VP1,2A) and complementing this plasmid with plasmid, pIM45-VP3, to produce particles. Production scheme for insertion of large peptides only in VP2 (bottom) involves insertion of peptide immediately after amino acid 138 in a plasmid that expresses only VP2 (pIM45-VP2A) and complementing this plasmid with plasmid, pIM45-VP1,3 to produce particles. FIG. 6B shows immunoblot of purified rAAV2-like particles produced by above production schemes with protein, leptin, inserted in VP1 and VP2 or only in VP2. FIG. 6B, left panel, shows immunoblot probed with antibody recognizing all three capsids proteins. FIG. 6B, right panel, shows immunoblot probed with antibody recognizing inserted peptide, leptin. Both panels: Lane 1: pIM45; Lane 2: pIM45-VP1,2A-Leptin/pIM45-VP3; Lane 3: pIM45-VP2A-Leptin/pIM45-VP1,3; Lane 4: pIM45-VP3 only; pIM45-VP1,3 only. FIG. 6C shows immunoblot of purified rAAV2-like particles produced by above production schemes with protein, GFP, inserted in VP1 and VP2 or only in VP2. FIG. 6C, left panel, shows immunoblot probed with antibody recognizing all three capsids proteins. FIG. 6C, right panel, shows immunoblot probed with antibody recognizing inserted peptide, GFP. Both panels: Lane 1: pIM45; Lane 2: pIM45-VP1,2A-GFP/pIM45-VP3; Lane 3: pIM45-VP2A-GFP/pIM45-VP1,3; Lane 4: pIM45-VP3 only; pIM45-VP1,3 only.

FIG. 7 shows Western blot of iodixanol virus stocks. Equal volumes of virus stock were separated by 10% SDS-PAGE and analyzed by Western blot using the B1 antibody.

FIG. 8 shows heparin-agarose binding profiles of mutant capsids. Approximately 5×10^{10} particles were applied to 500 μ l of heparin-agarose affinity matrix at a 100 mM NaCl concentration, washed extensively with the loading buffer, and bound capsids were eluted with 2 M NaCl. Pooled fractions were denatured and slot blotted onto nitrocellulose for immunodetection with mAb B1. For each mutant, L is the total amount of iodixanol

purified virus that was loaded onto the heparin agarose column; FT is the total virus that flowed through the column, W is the wash; E, eluate.

FIG. 9A and **FIG. 9B** show production and purification of AAV serotypes. **FIG. 9A** shows equivalent amounts of iodixanol purified AAV1, AAV2 and AAV5 were separated by 10% PAGE and analyzed by Western blot using the B1 antibody. **FIG. 9B** shows heparin-agarose binding properties of AAV2, AAV1 and AAV5. Abbreviations are the same as **FIG. 8**.

FIG. 10 shows particle-to-infectivity ratios of mutants relative to wild type. The particle-to-infectivity ratio for each mutant was calculated by dividing the average genomic titer by the average green cell assay titer (Table 2). The P/I ratio of each mutant was then normalized to wild type by dividing the P/I of each mutant by the P/I of wild type rAAV2, and the $\log_{10}$ value of the ratio was plotted. Wild type, therefore, equals one and is indicated by the dashed line. Grey bars, mutant viruses with infectivity comparable to wild type; Black bars, mutant viruses that are heparin binding deficient; White bars, mutant viruses with an undetermined block to infectivity; Asterisks indicate those mutants for which no green cells were scored. For these mutants the green cell assay titer used was the limit of detection in the assay. Thus, the log difference is a minimum estimate.

FIG. 11 shows GFP transduction ability of mutants in HeLa C12 cells. Cells were infected with wild type rAAV or mutant virus at an MOI=500 genomic particles and an Ad5 MOI=10 pfu per cell. Twenty-four hours post infection cells were fixed with 2% paraformaldehyde and the number of GFP positive cells was determined by FACS analysis.

FIG. 12A and **FIG. 12B** show binding and uptake of rAAV2 and R585A/R588A genomes in HeLa C12 cells. **FIG. 12A** shows 10^6 cells were infected with rAAV2 or R585/R588A at an MOI=100 or 1000 genome containing particles per cell, respectively. At the indicated times, infection media was removed and saved. The cells were washed and harvested, and Hirt DNA was extracted from both the infection media and the cell pellet. Southern analysis was performed using an $[\alpha\text{-}^{32}\text{P}]\text{-dATP}$ labeled GFP probe. **FIG. 12b** shows the percent bound/internalized DNA was calculated by dividing the total DNA present in both the media and the cell pellet by the amount bound/internalized for each time point. The average of three determinations is shown. Error bars indicate a standard deviation.

FIG. 13A, **FIG. 13B**, **FIG. 13C** and **FIG. 13D** show modifying the heparin binding properties of AAV5. **FIG. 13A** shows alignment of AAV2 amino acid residues 585 through 590 to residues predicted by amino acid alignment to be structurally equivalent in AAV5. **FIG. 13B** shows Western blot of iodixanol virus stocks. Equal volumes of virus were

separated by 10% SDS-PAGE and analyzed by Western blot using the B1 antibody. FIG. 13C shows novel heparin binding properties of AAV5-HS. Heparin-agarose binding was performed as described in FIG. 8. See FIG. 8 for abbreviations. FIG. 17D shows the log of the particle-to-infectivity ratio of the rAAV5 variants normalized to wild type rAAV2 as described in FIG. 10.

FIG. 14 shows an immunoslotblot of total capsid protein from novel production system following standard purification procedures. Immunoslotblot was probed with anti-VP1,2,3 monoclonal antibody. 1. pIM45/pIM45-VP0; 2. pIM45-VP1/pIM45-VP2,3; 3. pIM45-VP2acg/pIM45-VP1,3; 4. pIM45-VP2atg/pIM45-VP1,3; 5. pIM45-VP3/pIM45-VP1,2.

FIG. 15 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and system plasmid combinations. Numbering scheme is the same as described in FIG. 14. Equal volume aliquots from an iodixanol step gradient were with incubated with DNaseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabeled GFP probe.

FIG. 16 shows the *in vivo* transduction ability of recombinant AAV vectors produced using various system components. GFP fluorescence microscopy was performed on Hela C12 infected at an MOI of 1000 genomes/cell 24 hours post infection.

FIG. 17 shows the Immunoblot and dot blot autoradiograph of virions produced from pTR-UF5; pIM45-VP1,2; pIM45-VP1,3; and pIM45-VP2,3 plasmids following standard purification protocols. The capsid proteins VP1, VP2, and VP3 are indicated. No virions were obtained in 40% iodixanol fraction from plasmid pIM45-VP1,2.

FIG. 18 shows the *in vivo* transduction ability of recombinant AAV vectors containing only two capsid proteins. GFP fluorescence microscopy was performed on Hela C12/24 hours post infection.

FIG. 19 depicts an immunoblot of protein fractions collected from iodixanol purified passed over a heparin-agarose column. Immunoblot was probed with anti-VP1,2,3 monoclonal antibody. C, 5E+10 virus particles loaded directly onto blot, FT, flowthrough fraction, W, wash fraction, E, 2M NaCl fraction

FIG. 20 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and rAAV R585A, R588A. Equal volume aliquots from an iodixanol step gradient were with incubated with DNaseI, inactivated with EDTA, digested with proteinase K,

phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabeled GFP probe.

FIG. 21 shows the *in vivo* transduction ability of pTR-UF5 and R585A, R588A. GFP fluorescence microscopy was performed on Hela C12 and HEK 293 cells infected at an MOI of 1000 genomes/cell 24 hours post infection.

FIG. 22 shows a slot blot autoradiograph of an *in vivo* DNA tracking time course experiment of pTR-UF5, rAAV R585A, R588A. Media and cells infected with pTR-UF5 and rAAV R585A, R588A were collected at 1,4, and 20 hours post infection. Hirt DNA was extracted, transferred to nitrocellulose and probed with a radiolabeled GFP probe.

FIG. 23 shows a schematic diagram of the pIM45 vector showing the rep and cap sequences.

FIG. 24A and **FIG. 24B** show Western blot analysis of AAV capsid proteins in 293 cell lysates (**FIG. 24A**) and iodixanol purified virus stocks (**FIG. 24B**) following insertion of FKN or LEP peptides after residue 138 in the *Eag1/Mlu1* cloning site engineered in the VP1/2 overlap region. Equal volumes of lysates or virus stocks were separated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot using the B1 antibody. The diagram illustrates the position of the insertion of the E/M cloning site and the FKN and LEP ligands.

FIG. 25A, **FIG. 25B** and **FIG. 25C** show mutants that express only two capsid proteins. Western blot analysis of capsids in cell lysates produced from 293 cells transfected with mutants that eliminate expression of one of the three AAV capsid proteins. Equal volumes of extracts were separated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot using the B1 antibody. **FIG. 25A** shows the missense mutations within the start codons of the three capsid proteins (M1L, T138L, and M203L) are illustrated along with the capsid proteins expressed from each mutant on an SDS acrylamide gel blotted with B1 antibody. **FIG. 25B** shows the VP3-like proteins that result from read-through translation. A mutation in the normal VP3 start codon produces a truncated capsid protein, VP3a; mutations in the first two methionines (pM203,211L) produce a second truncated protein, VP3b; and mutations in the first three methionines (pM203,211,235L; pVP1,2) eliminate all VP3-like proteins. **FIG. 25C** shows an alternative approach to eliminating VP3 expression while maximizing VP2 expression. pVP1,2A contains a standard ATG start codon for VP2 instead of ACG, a T138M

mutation, thereby increasing VP2 expression and eliminating VP3 expression (compare pVP1,2A in FIG. 25C to pVP1,2 in FIG. 25B).

FIG. 26 shows mutants that express only a single capsid protein. Equal volumes of 293 cell extracts transfected with capsid mutants that express a single capsid protein were separated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot using B1 antibody. The diagram illustrates the missense mutation(s) in each construct.

FIG. 27A, FIG. 27B and FIG. 27C show which capsid mutants can make a virus particle. Western blot analysis of AAV virus purified by iodixanol step gradients as described below following transfection of the indicated capsid mutants into 293 cells. Equal volumes of the iodixanol fraction were separated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot using B1 antibody. **FIG. 27A** shows the effect of the M203L, M211L, and M235L mutations on particle formation. **FIG. 27B** shows particle formation from mutants that lack a specific capsid protein. **FIG. 27C** shows particle formation from mutants that express a single capsid protein.

FIG. 28A and FIG. 28B show complementation of mutants that make a single capsid protein. **FIG. 28A** shows Western blot analysis of AAV particles purified by iodixanol step gradients and heparin column chromatography following transfection of 293 cells with complementation groups described in Table 8. **FIG. 28B** shows Western blot analysis of iodixanol fractions of particles obtained from transfection with pVP2A, pVP3 or both plasmids. Equal volumes of purified virus stocks were separated by SDS-10% acrylamide gel electrophoresis and analyzed by Western blot using the B1 antibody.

FIG. 29A, FIG. 29B and FIG. 29C show capsid complementation strategy for creating particles with large peptide insertions in the VP1/VP2 overlap region. Western blot of equal volumes of iodixanol stocks of AAV-like particles containing FKN or LEP insertions at position 138. **FIG. 29A** shows a diagram of constructs used to complement insertions at amino acid 138 in both VP1 and VP2A or just VP2A. **FIG. 29B** shows particles with the FKN insertion were purified by iodixanol gradients and probed on SDS-10% polyacrylamide gels with anti-capsid (B1) antibody or anti-FKN antibody. **FIG. 29C** shows particles with the LEP insertion were purified by iodixanol gradients and probed on SDS-10% polyacrylamide gels with anti-capsid (B1) antibody or anti-LEP antibody.

FIG. 30A, FIG. 30B and FIG. 30C show capsid protein stoichiometry and infectivity of AAV virus stocks missing a capsid protein or containing a ligand insertion.

FIG. 30A shows Western blot of virus stocks purified by iodixanol gradients and heparin sulfate column chromatography. Approximately 1×10^{11} AAV-like particles were separated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot using the B1 antibody. FIG. 30B shows particle to infectivity ratios of AAV-like particles relative to that of pIM45. The particle to infectivity (P/I) ratio for each particle was calculated by dividing the average genomic titer by the average FCA titer (see Table 7). The P/I ratio for each type of virus was then normalized to that of wild type virus (pIM45) by dividing the P/I of each AAV-like particle by the P/I of pIM45, and the log10 value of the ratio was plotted. The wild type pIM45 ratio equals zero and is indicated by the dashed line. Grey bars, particles with infectivity comparable to pIM45 (within 1 log); white bars, particles with significantly reduced infectivity (1-4 logs lower infectivity), black bars, particles that were essentially non-infectious (> 4 logs). FIG. 30C shows Western blot of approximately 1×10^{11} AAV-like particles with GFP inserted in the capsid. Virus samples were purified as in FIG. 30A above, fractionated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot using the B1 antibody.

FIG. 31 shows time course of VP1,2A-GFP + VP3 particle trafficking following infection in the absence (top panel) and presence (bottom panel) of Ad 5. HeLa cells were infected with AAV containing a GFP insertion at an MOI of $10,000 \pm$ Ad 5 at an MOI of 20. Vectors remained on the cells for the duration of the time course. The input capsids appear green from the native GFP fluorescence of the capsid, the nuclei are stained red with propidium iodide and the early endosomal antigen, EER1, is stained blue.

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

4.1 RAAV TYPE 2

Adeno-associated virus 2 (AAV) (Muzyczka and Berns, 2001) requires the assembly of 60 individual structural proteins into a non-enveloped, T-1 icosahedral lattice capable of protecting a 4.7 kb single-stranded DNA genome (Kronenberg *et al.*, 2001; Xie *et al.*, 2002). Purified infectious AAV particles contain three major structural proteins designated VP1, VP2 and VP3 (87, 73 and 62 kDa, respectively) in an approximate ratio of 1:1:18 (Buller and Rose, 1978). The anti-parallel β -barrel topology of these capsid proteins results in a particle with a defined tropism (Kern *et al.*, 2003; Opie *et al.*, 2003; Qing *et al.*, 1999; Summerford *et al.*, 1999; Summerford and Samuski, 1998) that is highly resistant to degradation.

The three AAV capsid proteins are produced in an overlapping fashion from the *cap* ORF using alternative mRNA splicing of the transcript and alternative translational start codon usage (Becerra *et al.*, 1988 Becerra *et al.*, 1985; Cassinotti *et al.*, 1988; Janik *et al.*, 1984; McPherson and Rose, 1983; Rose *et al.*, 1971; Trempe and Carter, 1988; Weger *et al.*, 1997). A common stop codon is employed for all three proteins (Srivastava *et al.*, 1983). Correct capsid protein stoichiometry is maintained by translating VP1 from an ATG start codon (amino acid M1) on the 2.4 kb mRNA (Becerra *et al.*, 1988; Cassinotti *et al.*, 1988; Trempe and Carter, 1988), while VP2 and VP3 arise from the 2.3 kb mRNA, using a weaker ACG start codon for VP2 production and read-through translation to the next available ATG codon for the production of the most abundant capsid protein, VP3 (amino acids T138 and M203, respectively) (Becerra *et al.*, 1985; Muralidhar *et al.*, 1994).

The specific roles for the individual capsid proteins in the assembly process and the absolute requirements for each in the formation of a functional virus particle are unclear. Studies of the viral life cycle in the absence of capsid protein expression (Hermonat *et al.*, 1984; Smuda and Carter, 1991; Tratschin *et al.*, 1984; Vincent *et al.*, 1997) and reports of capsid intermediates that accumulate during AAV infection (Dubielzig *et al.*, 1999; Hunter and Samulski, 1992; Kube *et al.*, 1997; Prasad and Trempe, 1995; Wistuba *et al.*, 1997; Wistuba *et al.*, 1995) indicate that these proteins are required for the accumulation of single stranded genomes and clearly show that the assembly process occurs in the nucleus. Absence of the largest capsid protein VP1, or deletion of the N-terminal sequence unique to VP1, leads to assembly of low infectivity particles (*lip*) (Hermonat *et al.*, 1984;

Tratschin *et al.*, 1984; Wu *et al.*, 2000). This phenotype has been shown to be due to the absence of a phospholipase activity in the amino acid sequence unique to VP1 (Girod *et al.*, 1999; Zadori *et al.*, 2001). Some evidence also suggests that expression of either of the less abundant proteins, VP1 or VP2, is necessary for assembly of empty or full (genome containing) particles (Hoque *et al.*, 1999; Ruffing *et al.*, 1992; Steinbach *et al.*, 1997; Wistuba *et al.*, 1997). Site-directed missense mutagenesis of the individual capsid protein start codons or the expression of separate capsid protein genes suggests that empty or full particles are obtained only if VP3 is co-expressed with VP1 or VP2 (Hoque *et al.*, 1999; Muralidhar *et al.*, 1994; Steinbach *et al.*, 1997; Wistuba *et al.*, 1997). AAV capsid protein expression in SF9 cells (Ruffing *et al.*, 1992) also suggests an essential role for VP2 in particle formation. The requirement for either VP1 or VP2 for capsid assembly seems to correlate with a lower nuclear localization of VP3, the most abundant capsid protein (Hoque *et al.*, 1999; Ruffing *et al.*, 1992; Steinbach *et al.*, 1997). However, a more recent insertional mutagenesis analysis of the *cap* ORF (Rabinowitz *et al.*, 1999) has reported the formation of a particle composed only of VP3, and studies in the absence of Ad helper function and packageable AAV genomes have shown that intact virus like particles can be formed with VP3 alone provided that the VP3 is fused to a nuclear localization signal (Hoque *et al.*, 1999). Finally, studies of capsid assembly in insect cells, when the three capsid proteins were expressed from separate constructs in the absence of viral DNA or helper virus, suggest that VP1+VP3 or VP1+VP2 or VP2 alone can form virus like particles (Ruffing *et al.*, 1992), while similar studies in HeLa cells suggest that VP1 or VP2 alone, but not VP3, could form intact particles (Steinbach *et al.*, 1997). Thus, the absolute requirement for each capsid protein in the formation of intact particles has not been completely resolved.

4.2 RAAV CAPSID PROTEINS

Supramolecular assembly of 60 individual capsid protein subunits into a non-enveloped, T-1 icosahedral lattice capable of protecting a 4.7 kb single-stranded DNA genome is a critical step in the life-cycle of the helper-dependent human parvovirus, adeno-associated virus2 (AAV2). The mature 20 nm diameter AAV2 particle is composed of three structural proteins designated VP1, VP2, and VP3 (molecular masses of 87, 73, and 62 kDa respectively) in a ratio of 1:1:18. Based on its symmetry and these molecular weight estimates, of the 60 capsid proteins comprising the particle, three are VP1 proteins, three are

VP2 proteins, and fifty-four are VP3 proteins. The employment of three structural proteins makes AAV serotypes unique among parvoviruses, as all others known package their genomes within icosahedral particles composed of only two capsid proteins. The anti-parallel β -strand barreloid arrangement of these 60 capsid proteins results in a particle with a defined tropism that is highly resistant to degradation.

The AAV2 genome contains two large open reading frames (ORF), rep and cap, flanked by inverted terminal repeats. The AAV2 capsid proteins are produced in an overlapping fashion from the cap ORF; arising through alternative mRNA splicing of the transcript (initiated at the p40 promoter), with subsequent alternative translational start codon usage. A common stop codon is employed for all three capsid proteins. Correct capsid protein stoichiometry is maintained by translating VP1 from the 2.4 KB mRNA, while VP2 and VP3 arise from the 2.3-kB mRNA using a weaker ACG start codon for VP2 protein production with resultant read-through translation for the production of the VP3 protein. Differing only in the length of their N-terminus, these proteins are produced such that the amino acid sequence of VP3 is contained within the significantly less abundant and longer VP1 and VP2 proteins. As such, VP1's unique 137 amino acid N-terminal extension of VP2 contains a phospholipase enzymatic activity important for viral infectivity. Similarly, VP2 extends the N-terminus of VP3 by 64 amino acids with this VP1/VP2 overlap region possessing a putative nuclear localization signal (NLS) involved in the nuclear translocation of VP2. The VP3 region common to all three capsid proteins contains the critical β -barrel structural motifs characteristic of all parvoviruses and particle surface loops involved in determining viral tropism.

While specific activities have been attributed to regions of an individual AAV2 capsid protein, the role of each capsid protein in the structural formation of the particle is less clear. Early studies in which all AAV2 capsid expression was eliminated revealed that capsid protein expression is required for the accumulation of single stranded genomes. It follows that AAV2 particle assembly occurs within the nucleus, and a putative NLS for VP2 has been localized to the VP1/VP2 overlap region in transfected COS cells.

In the absence of VP1 expression, this study suggested a major role of VP2 is the nuclear localization of VP3. However, since VP1 was deleted in this study, one cannot rule out that VP1 has the ability to nuclear localize VP3. Site-directed missense mutagenesis of the individual capsid proteins' start codons suggested that infectious particles are obtained only when all three capsid proteins are present. In contrast, later genetic analysis demonstrated that in the absence of VP1, VP2 and VP3 are able to encapsidate progeny

genomes. Similarly, *in vitro* assembly of purified individual AAV capsid proteins demonstrated that VP2 and VP3 could form an AAV2-like particle. Baculovirus expression of the AAV2 capsid proteins within SF9 cells suggests an absolute requirement for VP2, although this study failed to eliminate VP3-like fragments produced by the VP2-baculovirus. However, it is feasible that studies of AAV2 assembly in baculovirus have subtle differences with particles assembled in mammalian cells.

An examination of the assembly process of the related autonomous canine parvovirus, CPV, in baculovirus observed significantly more aggregation of capsid proteins in insect cells. In addition, the results of the baculovirus and NLS studies have the caveat that they were performed in the absence of AAV2 Rep proteins, Ad helper gene functions, and a replicating AAV2 genome. Furthermore, the p40 promoter in these studies does not control AAV2 capsid protein expression, resulting in altered stoichiometry of the available capsid protein pool. Indeed, the above concerns seem warranted, as a recent insertional mutagenesis study of the AAV2 cap ORF, using standard AAV2 production protocols, reported the purification of an AAV2-like particle composed of only the VP3 protein. Therefore, despite the uncertainty of the precise role of VP1 and VP2 in particle formation, the evidence thus far suggests that the VP3 protein is absolutely required for the formation of an AAV2 particle. Finally, co localization studies of AAV2 assembly in 293 cells demonstrated an interaction of AAV2 Rep and capsid proteins with Ad proteins and the replicating genome in the nucleus, thus, supporting a current model of AAV2 assembly which proposes nucleoplasmic formation of empty particles with subsequent maturation of the particle as a result of Rep 52/40 mediated translocation of capsid protein associated single stranded genomes into the preformed particles.

4.3 GENETIC MODIFICATION OF RAAV CAPSID PROTEINS

Great interest in the assembly, structure, and mutability of the AAV2 particle results from its promise as a recombinant gene delivery vehicle (rAAV2) *in vivo*. Essential to the clinical development of rAAV2 vectors for gene therapy is the ability to target specific tissue types. Manipulation of the rAAV2 particle in order to control its cellular receptor interactions is essential for vector targeting. The feasibility of various targeting strategies based on AAV cap ORF mutagenesis is currently an area of active investigation. A better understanding of the AAV2 particle surface architecture through systematic scanning-alanine and insertional mutagenesis of the AAV cap ORF and recent publication of the AAV2 crystal

structure has identified several amino acid regions on the surface of the particle that tolerate sequence alteration without loss of capsid stability or integrity.

However, small changes in charge, sequence, and/or position of the mutation can result in dramatic changes in the mutant particle phenotype. One limitation in sequence mutation of the overlapping cap ORF is that mutation of only one capsid protein across its entire sequence is currently not possible. The full potential in manipulation of the particle is not reached with direct alteration of regions of capsid overlap. Predicted surface regions of capsid overlap leading to defective phenotypes upon mutagenesis may allow production of viable particles if such mutations were only in one or two of the capsid proteins. An additional degree of flexibility in modifying the rAAV2 particle would result from the ability to mutate the entire coding region of a specific capsid protein without altering the remaining two capsid proteins. Indeed, while mutations in the C-terminus of the VP3 region have been reported to be completely defective in particle formation following insertion of HA and 6xHis tags into the overlapping cap ORF, a recent report focusing on the purification of rAAV2 particles demonstrated that the C-terminus of VP3 is capable of accepting a 6xHis tag if VP1 and VP2 are not altered. This rAAV2 production strategy involved expressing VP1 and VP2 from one construct, and expressing the VP3-6xHis fusion protein from a CMV promoter in a second plasmid. In the absence of the isolation of a specific capsid protein's expression, the N-terminal 137 amino acids of VP1 are the only region of the cap ORF where mutations are restricted to a single capsid protein. Successful insertions within this region have included HA and serpin. The VP1/VP2 overlap region (amino acid 138-202) also has been receptive to sequence modification. Insertions in this region have included HA, serpin and luteinizing hormone receptor ligand sequences immediately following amino acid 138 in the cap ORF.

The success of inserting sequences to the VP1 and VP1/VP2 regions may be due in part to less disruption of the integrity of the particle compared to insertion in the VP3 region of capsid overlap (amino acid 203-735). It is important to note that these mutant particles would require further mutation of the putative heparin-binding motif to restrict infection to the target cell. Not surprisingly, since it is the longest region of capsid protein overlap, contains many critical structural motifs, and targeting sequences in this region have 60 representatives in the rAAV particle, mutations in the VP3 region of the AAV2 cap ORF have resulted in the highest number of defective phenotypes. Yet, one location within the VP3 region has received much attention for the successful insertion of small targeting sequences in all three capsid proteins (amino acid 587). One major advantage of targeting

insertions to this position is that the resultant mutant particle also has lost the ability to bind its native receptor. Viable mutations in the VP3 region of the cap ORF have been restricted in size (<30 amino acids).

One caveat of creating genetically-targeted rAAV2 particles, is the consideration that many cell surface receptors have ligands whose coding sequence are much larger than those successfully inserted directly into the overlapping cap ORF. Due to the modest size of this ORF (~2kB), the insertion of larger peptide sequences into the capsid coding sequences may result in serious disruption of splicing, read-through translation, capsid structure and/or stability. The insertion of large sequences into the rAAV2 particle have been limited to a study involving the fusion of the CD34 single chain antibody coding sequence with the N-termini of the individual capsid proteins following isolation of their expression to separate CMV promoters. Viable CD34-retargeted rAAV particles of extremely low titer were produced only when this fusion was to VP2 protein, and co-expression of wild-type VP2 protein was required. Nonetheless, the fusion of large peptide sequences to the N-terminus of VP2 does not interfere with the incorporation of this capsid protein into the rAAV2 particle.

4.4 RAAV VP2 CAPSID CAN TOLERATE LARGE PEPTIDE INSERTIONS

Direct insertion of amino acid sequences into the adeno-associated virus type 2 (AAV) capsid open reading frame (*cap* ORF) is one strategy currently being developed for retargeting this prototypical gene therapy vector. While this approach has successfully resulted in the formation of AAV particles that have expanded or retargeted viral tropism, the inserted sequences have been relatively short, linear receptor binding ligands. Since many receptor/ligand interactions involve non-linear, conformation dependent binding domains, the insertion of full length peptides into the AAV *cap* ORF was investigated. To minimize disruption of critical VP3 structural domains, insertions have been confined to residue 138 within the VP1/2 overlap, which has been shown to be on the surface of the particle following insertion of smaller epitopes. The insertion of coding sequences for the 8 kDa chemokine binding domain of rat fractalkine (FKN, CX3CL1), the 18 kDa human hormone, leptin (LEP), and the 30 kDa green fluorescent protein (GFP) after residue 138 failed to form particles due to the loss of VP3 expression. To test the ability to complement these insertions with the missing capsid proteins *in trans*, a system has been designed and utilized for producing AAV vectors in which expression of one capsid protein

is isolated and combined with the remaining two capsid proteins expressed separately. Such an approach allows for genetic modification of a specific capsid protein across its entire coding sequence leaving the remaining capsid proteins unaffected.

Examination of particle formation from the individual components of the system has revealed that genome containing particles formed as long as the VP3 capsid protein was present, and demonstrated that the VP2 capsid protein is non-essential for viral infectivity. Viable particles composed of all three capsid proteins were obtained from the capsid complementation groups regardless of which capsid proteins were supplied separately *in trans*. Significant over-expression of VP2 resulted in the formation of particles with altered capsid protein stoichiometry. Using this system the inventors have successfully obtained nearly wild-type levels of recombinant AAV-like particles with large ligands inserted after residue 138 in VP1 and VP2, or in VP2 exclusively. While insertions at residue 138 in VP1 significantly decreased infectivity, insertions at residue 138 that were exclusively in VP2 had minimal effect on viral assembly or infectivity. Finally, insertion of GFP into VP1 and VP2 resulted in a particle whose trafficking could be temporally monitored using confocal microscopy. Thus, the invention has produced a method that can be used to insert large (up to 30 kDa) peptide ligands into the AAV particle. This system allows greater flexibility than current approaches in genetically manipulating the composition of the AAV particle, and, in particular, may allow vector retargeting to alternative receptors requiring interaction with full length conformation dependent peptide ligands.

4.5 WILD-TYPE AAV2 BINDS TO HEPARAN SULFATE PROTEOGLYCAN

The adeno-associated virus type-2 (AAV2) uses heparan sulfate proteoglycan (HSPG) as its primary cellular receptor. In order to identify amino acids within the capsid of AAV2 that contribute to HSPG association, biochemical information about heparin/heparin sulfate (HS), AAV serotype protein sequence alignments, and data from previous capsid studies was used to select residues for mutagenesis. In the present invention, charged-to-alanine substitution mutagenesis was performed on individual and combinations of basic residues for the production and purification of recombinant viruses that contained a GFP reporter gene cassette. Intact capsids were assayed for their ability to bind to heparin-agarose *in vitro* and virions that packaged DNA were assayed for their ability to transduce normally permissive cell lines. It was found that mutation of arginine residues at position 585 or 588

eliminated binding to heparin-agarose. Mutation of residues R484, R487, and K532 showed partial binding to heparin-agarose. A general correlation between heparin-agarose binding and infectivity was observed as measured by GFP transduction; however, a subset of mutants that partially bound heparin-agarose (R484A and K532A) were completely non-infectious, suggesting that they had additional blocks to infectivity that were unrelated to heparin binding. Conservative mutation of positions R585 and R588 to lysine slightly reduced heparin-agarose binding, and had comparable effects on infectivity. Substitution of AAV2 residues 585 through 590 into a location predicted to be structurally equivalent in AAV5 generated a hybrid virus that bound to heparin-agarose efficiently, was able to package DNA, but was non-infectious. Taken together, these suggest that residues R585 and R588 are primarily responsible for heparin sulfate binding and mutation of these residues has little effect on other aspects of the viral life cycle.

Computer modeling using the AAV2 VP3 atomic coordinates revealed that residues which contribute to heparin binding form a cluster of five basic amino acids on the surface of each three-fold axis of symmetry related spike. Three other kinds of mutants were found as well. Mutants, R459A, H509A and H526A/K527A bound heparin as well as wild type but were defective for transduction. Another mutant, H358A, was defective for capsid assembly. Finally, a mutant R459A produced significantly lower levels of full capsids, suggesting a packaging defect.

4.6 PHARMACEUTICAL COMPOSITIONS

The genetic constructs of the present invention may be prepared in a variety of compositions, and may also be formulated in appropriate pharmaceutical vehicles for administration to human or animal subjects. The AAV molecules of the present invention and compositions comprising them provide new and useful therapeutics for the treatment, control, and amelioration of symptoms of a variety of disorders. Moreover, pharmaceutical compositions comprising one or more of the nucleic acid compounds disclosed herein, provide significant advantages over existing conventional therapies – namely, (1) their reduced side effects, (2) their increased efficacy for prolonged periods of time, (3) their ability to increase patient compliance due to their ability to provide therapeutic effects following as little as a single administration of the selected therapeutic AAV composition to affected individuals. Exemplary pharmaceutical compositions and methods for their administration are discussed in significant detail hereinbelow.

The invention also provides compositions comprising one or more of the disclosed vectors, expression systems, virions, viral particles; or mammalian cells. As described hereinbelow, such compositions may further comprise a pharmaceutical excipient, buffer, or diluent, and may be formulated for administration to an animal, and particularly a human being. Such compositions may further optionally comprise a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle, or may be otherwise formulated for administration to the cells, tissues, organs, or body of a mammal in need thereof. Such compositions may be formulated for use in therapy, such as for example, in the amelioration, prevention, or treatment of conditions such as peptide deficiency, polypeptide deficiency, tumor, cancer or other malignant growth, neurological dysfunction, autoimmune diseases, lupus, cardiovascular disease, pulmonary disease, ischemia, stroke, cerebrovascular accidents, diabetes and diseases of the pancreas, neural diseases, including Alzheimer's, Huntington's, Tay-Sach's, and Parkinson's diseases, memory loss, trauma, motor impairment, and the like, as well as biliary, renal or hepatic disease or dysfunction, as well as musculoskeletal diseases including, for example, arthritis, cystic fibrosis (CF), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), muscular dystrophy (MD), and such like, to name only a few.

In certain embodiments, the present invention concerns formulation of one or more of the rAAV compositions disclosed herein in pharmaceutically acceptable solutions for administration to a cell or an animal, either alone or in combination with one or more other modalities of therapy, and in particular, for therapy of human cells, tissues, and diseases affecting man.

It will also be understood that, if desired, nucleic acid segments, RNA, DNA or PNA compositions that express one or more of therapeutic gene products may be administered in combination with other agents as well, such as, *e.g.*, proteins or polypeptides or various pharmaceutically-active agents, including one or more systemic or topical administrations of therapeutic polypeptides, biologically active fragments, or variants thereof. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The rAAV compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA, DNA, or PNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically-useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In certain circumstances it will be desirable to deliver the AAV vector-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intraocularly, intravitreally, parenterally, subcutaneously, intravenously, intracerebro-ventricularly, intramuscularly, intrathecally, orally, intraperitoneally, by oral or nasal inhalation, or by direct injection to one or more cells, tissues, or organs by direct injection. The methods of administration may also include those modalities as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as freebase or pharmacologically acceptable salts may be prepared in sterile water and may also suitably mixed with one or more surfactants, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms of the AAV-based viral compositions suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium

containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active AAV vector-delivered therapeutic polypeptide-encoding DNA fragments in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The AAV vector compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the

free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human, and in particular, when administered to the human eye. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The amount of AAV compositions and time of administration of such compositions will be within the purview of the skilled artisan having benefit of the present teachings. It is likely, however, that the administration of therapeutically-effective amounts of the disclosed compositions may be achieved by a single administration, such as for example, a single injection of sufficient numbers of infectious particles to provide therapeutic benefit to the patient undergoing such treatment. Alternatively, in some circumstances, it may be desirable to provide multiple, or successive administrations of the AAV vector compositions, either over a relatively short, or a relatively prolonged period of time, as may be determined by the medical practitioner overseeing the administration of such compositions. For example, the number of infectious particles administered to a mammal may be on the order of about 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} , or even higher, infectious particles/ml given either as a single dose, or divided into two or

more administrations as may be required to achieve therapy of the particular disease or disorder being treated. In fact, in certain embodiments, it may be desirable to administer two or more different AAV vector compositions, either alone, or in combination with one or more other therapeutic drugs to achieve the desired effects of a particular therapy regimen.

4.7 LIPOSOME-, NANOCAPSULE-, AND MICROPARTICLE-MEDIATED DELIVERY

In certain embodiments, the inventors contemplate the use of liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered gene therapy compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically incorporated herein by reference in its entirety). Further, various methods of liposome and liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, 1990; Muller *et al.*, 1990). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs (Heath and Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta and Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller and Baltimore, 1984), transcription factors and allosteric effectors (Nicolau and Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*,

1985a; 1985b; Coune, 1988; Sculier *et al.*, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori and Fukatsu, 1992).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μ m. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1988), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles

(LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Targeting is generally not a limitation in terms of the present invention. However, should specific targeting be desired, methods are available for this to be accomplished. Antibodies may be used to bind to the liposome surface and to direct the antibody and its drug contents to specific antigenic receptors located on a particular cell-type surface. Carbohydrate determinants (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have

potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous injection of liposomal preparations would be used, but other routes of administration are also conceivable.

Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the AAV vector-based polynucleotide compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be easily made, as described (Couvreur *et al.*, 1980; Couvreur, 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

4.8 ADDITIONAL MODES OF DELIVERY

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the disclosed rAAV vector based polynucleotide compositions to target cells or selected tissues and organs of an animal, and in particular, to cells, organs, or tissues of a vertebrate mammal, and more particularly, to a primate, such as a human being. Sonophoresis (*i.e.*, ultrasound) has been used and described in U. S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U. S. Patent 5,779,708), microchip devices (U. S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U. S. Patent 5,770,219 and U. S. Patent 5,783,208) and feedback-controlled delivery (U. S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

4.9 PROMOTERS AND ENHANCERS

Recombinant AAV vectors, and compositions and pharmaceutical formulations comprising them form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression includes transcription of the nucleic acid, for example, to generate a biologically-active therapeutic agent(s), such as, for example, one or more peptides,

polypeptides, proteins, enzymes, or an antisense polynucleotide or oligonucleotide, or catalytic RNA molecules such as ribozymes, from a selected nucleic acid segment that encodes the therapeutic agent or agents.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of one or more promoter and/or enhancer elements that are capable of directing synthesis of the encoded therapeutic in a selected cell into which the vectors have been introduced. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "operably positioned" "operably linked" "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the selected nucleic acid segment encoding the therapeutic agent.

In certain embodiments, it is contemplated that certain advantages will be gained by positioning the coding polynucleotide segment under the control of at least a first recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with the gene in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from bacterial, viral, eukaryotic, or mammalian cells.

Naturally, it will be desirable to employ a promoter that effectively directs the expression of the encoded therapeutic agent in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced polynucleotide segment, or the promoters may direct tissue- or cell-specific expression of the therapeutic constructs, such as, for example, an islet cell- or pancreas-specific promoter such as the insulin promoter.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the serpin or cytokine-polypeptide encoding nucleic acid segment in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter, such as a CMV or an HSV promoter. In certain aspects of the invention, β -actin, and in particular, chicken β -actin promoters have been shown to be particularly preferred for certain embodiments of the invention.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. A variety of promoter elements have been described in Tables 1 and 2 that may be employed, in the context of the present invention, to regulate the expression of the present serpin or cytokine-encoding nucleic acid segments comprised within the recombinant AAV vectors of the present invention.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of

a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1
ILLUSTRATIVE PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCE
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto and Baltimore, 1989; Redondo <i>et al.</i> ; 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-Dra	Sherman <i>et al.</i> , 1989
β -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989
Muscle Creatine Kinase	Jaynes <i>et al.</i> , 1988; Horlick and Benfield, 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Omitz <i>et al.</i> , 1987
Metallothionein	Karin <i>et al.</i> , 1987; Culotta and Hamer, 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987

PROMOTER/ENHANCER	REFERENCE
Albumin Gene	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
α -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β -Globin	Trudel and Constantini, 1987
e-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh <i>et al.</i> , 1990
α_1 -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy SV40	Klamut <i>et al.</i> , 1990 Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleight and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander and Haseltine, 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Chol <i>et al.</i> , 1988; Reisman and Rotter, 1989

PROMOTER/ENHANCER	REFERENCE
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky and Botchan, 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens and Hentschel, 1987
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber and Cullan, 1988; Jakobovits <i>et al.</i> , 1988; Feng and Holland, 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp and Marciniak, 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

TABLE 2
INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger and Karin, 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors and Varmus, 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
β -Interferon	poly(rI)x poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b

ELEMENT	INDUCER	REFERENCES
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
α -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blonar <i>et al.</i> , 1989
HSP70	Ela, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

As used herein, the terms “engineered” and “recombinant” cells are intended to refer to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active serpin or cytokine polypeptide or a ribozyme specific for such a biologically-active serpin or cytokine polypeptide product, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous DNA segment. Engineered cells are thus cells having DNA segment introduced through the hand of man.

To express a biologically-active serpin or cytokine encoding gene in accordance with the present invention one would prepare an rAAV expression vector that comprises a biologically-active serpin or cytokine polypeptide-encoding nucleic acid segment under the control of one or more promoters. To bring a sequence “under the control of” a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides “downstream” of (*i.e.*, 3' of) the chosen promoter. The “upstream” promoter stimulates transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of “recombinant expression” in this context. Particularly preferred recombinant vector constructs are those that comprise an rAAV vector. Such vectors are described in detail herein.

4.10 MUTAGENESIS AND PREPARATION OF MODIFIED NUCLEOTIDE COMPOSITIONS

In certain embodiments, it may be desirable to prepared modified nucleotide compositions, such as, for example, in the generation of the nucleic acid segments that encode

either parts of the AAV vector itself, or the promoter, or even the therapeutic gene delivered by such rAAV vectors. Various means exist in the art, and are routinely employed by the artisan to generate modified nucleotide compositions.

Site-specific mutagenesis is a technique useful in the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector that includes within its sequence a DNA sequence encoding the desired ribozyme or other nucleic acid construct. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected nucleic acid sequences using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

4.11 NUCLEIC ACID AMPLIFICATION

In certain embodiments, it may be necessary to employ one or more nucleic acid amplification techniques to produce the nucleic acid segments of the present invention. Various methods are well-known to artisans in the field, including for example, those techniques described herein:

Nucleic acid, used as a template for amplification, may be isolated from cells contained in the biological sample according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to the ribozymes or conserved flanking regions are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even *via* a system using electrical or thermal impulse signals (*e.g.*, Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best-known amplification methods is the polymerase chain reaction (referred to as PCR[™]), which is described in detail in U. S. Patent No. 4,683,195, U. S. Patent No. 4,683,202 and U. S. Patent No. 4,800,159 (each of which is incorporated herein by reference in its entirety).

Briefly, in PCR[™], two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq*

polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.* (1989). Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in Int. Pat. Appl. Publ. No. WO 90/07641 (specifically incorporated herein by reference). Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, and incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Q β Replicase (Q β R), described in Int. Pat. Appl. No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α -thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA), described in U. S. Patent Nos. 5,455,166, 5,648,211, 5,712,124 and 5,744,311, each incorporated herein by reference, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for

amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in Int. Pat. Appl. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR[™]-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, Int. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer that has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA

("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, Int. Pat. Appl. Publ. No. WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR™" (Frohman, 1990, specifically incorporated herein by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide," thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (see *e.g.*, Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U. S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

4.12 METHODS OF NUCLEIC ACID DELIVERY AND DNA TRANSFECTION

In certain embodiments, it is contemplated that one or more RNA, DNA, PNAs and/or substituted polynucleotide compositions disclosed herein will be used to transfect an appropriate host cell. Technology for introduction of PNAs, RNAs, and DNAs into cells is well known to those of skill in the art.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Wong and Neumann, 1982; Fromm

et al., 1985; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984; Suzuki *et al.*, 1998; Vanbever *et al.*, 1998), direct microinjection (Capecchi, 1980; Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Takakura, 1998) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990; Klein *et al.*, 1992), and receptor-mediated transfection (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

4.13 EXPRESSION VECTORS

The present invention contemplates a variety of AAV-based expression systems, and vectors. In one embodiment the preferred AAV expression vectors comprise at least a first nucleic acid segment that encodes a therapeutic peptide, protein, or polypeptide. In another embodiment, the preferred AAV expression vectors disclosed herein comprise at least a first nucleic acid segment that encodes an antisense molecule. In another embodiment, a promoter is operatively linked to a sequence region that encodes a functional mRNA, a tRNA, a ribozyme or an antisense RNA.

As used herein, the term “operatively linked” means that a promoter is connected to a functional RNA in such a way that the transcription of that functional RNA is controlled and regulated by that promoter. Means for operatively linking a promoter to a functional RNA are well known in the art.

The choice of which expression vector and ultimately to which promoter a polypeptide coding region is operatively linked depend directly on the functional properties desired, *e.g.*, the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is capable of directing the expression of the functional RNA to which it is operatively linked.

RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

A variety of methods have been developed to operatively link DNA to vectors *via* complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and

DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

4.14 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes to the structure of the polynucleotides and polypeptides of wild-type rAAV vectors to provide the improved rAAV virions as described in the present invention to obtain functional viral vectors that possess desirable characteristics, particularly with respect to improved delivery of therapeutic gene constructs to selected mammalian cell, tissues, and organs for the treatment, prevention, and prophylaxis of various diseases and disorders, as well as means for the amelioration of symptoms of such diseases, and to facilitate the expression of exogenous therapeutic and/or prophylactic polypeptides of interest *via* rAAV vector-mediated gene therapy. As mentioned above, one of the key aspects of the present invention is the creation of one or more mutations into specific polynucleotide sequences that encode one or more of the therapeutic agents encoded by the disclosed rAAV constructs. In certain circumstances, the resulting polypeptide sequence is altered by these mutations, or in other cases, the sequence of the polypeptide is unchanged by one or more mutations in the encoding polynucleotide to produce modified vectors with improved properties for effecting gene therapy in mammalian systems.

When it is desirable to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, second-generation molecule, the amino acid changes may be achieved by changing one or more of the codons of the encoding DNA sequence, according to Table 3.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the polynucleotide sequences disclosed herein, without appreciable loss of their biological utility or activity.

TABLE 3

Amino Acids		Codons							
Alanine	Ala	A	GCA	GCC	GCG	GCU			
Cysteine	Cys	C	UGC	UGU					
Aspartic acid	Asp	D	GAC	GAU					
Glutamic acid	Glu	E	GAA	GAG					
Phenylalanine	Phe	F	UUC	UUU					
Glycine	Gly	G	GGA	GGC	GGG	GGU			
Histidine	His	H	CAC	CAU					
Isoleucine	Ile	I	AUA	AUC	AUU				
Lysine	Lys	K	AAA	AAG					
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU	
Methionine	Met	M	AUG						
Asparagine	Asn	N	AAC	AAU					
Proline	Pro	P	CCA	CCC	CCG	CCU			
Glutamine	Gln	Q	CAA	CAG					
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU	
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU	
Threonine	Thr	T	ACA	ACC	ACG	ACU			
Valine	Val	V	GUA	GUC	GUG	GUU			
Tryptophan	Trp	W	UGG						
Tyrosine	Tyr	Y	UAC	UAU					

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine

(-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those that are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those that are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take several of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.15 THERAPEUTIC AND DIAGNOSTIC KITS

The invention also encompasses one or more of the modified rAAV vector compositions described herein together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular rAAV-polynucleotide delivery formulations, and in the preparation of therapeutic

agents for administration to a mammal, and in particular, to a human. In particular, such kits may comprise one or more of the disclosed rAAV compositions in combination with instructions for using the viral vector in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include murines, bovines, equines, porcines, canines, and felines. The composition may include partially or significantly purified rAAV compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

Therapeutic kits may also be prepared that comprise at least one of the compositions disclosed herein and instructions for using the composition as a therapeutic agent. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed rAAV composition(s) may be placed, and preferably suitably aliquoted. Where a second therapeutic polypeptide composition is also provided, the kit may also contain a second distinct container means into which this second composition may be placed. Alternatively, the plurality of therapeutic biologically active compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container means. The kits of the present invention will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.16 RIBOZYMES AND CATALYTIC RNA MOLECULES

As mentioned above, one aspect of the invention concerns the use of the modified capsid vectors to deliver catalytic RNA molecules (ribozymes) to selected mammalian cells and tissues to effect a reduction or elimination of expression of one or more native DNA or mRNA molecules, so as to prevent or reduce the amount of the translation product of such mRNAs. Ribozymes are biological catalysts consisting of only RNA. They promote a variety of reactions involving RNA and DNA molecules including site-specific cleavage, ligation, polymerization, and phosphoryl exchange (Cech, 1989; Cech, 1990). Ribozymes fall into three broad classes: (1) RNase P, (2) self-splicing introns, and (3) self-cleaving viral agents. Self-

cleaving agents include hepatitis delta virus and components of plant virus satellite RNAs that sever the RNA genome as part of a rolling-circle mode of replication. Because of their small size and great specificity, ribozymes have the greatest potential for biotechnical applications. The ability of ribozymes to cleave other RNA molecules at specific sites in a catalytic manner has brought them into consideration as inhibitors of viral replication or of cell proliferation and gives them potential advantage over antisense RNA. Indeed, ribozymes have already been used to cleave viral targets and oncogene products in living cells (Koizumi *et al.*, 1992; Kashani-Sabet *et al.*, 1992; Taylor and Rossi, 1991; von-Weizsacker *et al.*, 1992; Ojwang *et al.*, 1992; Stephenson and Gibson, 1991; Yu *et al.*, 1993; Xing and Whitton, 1993; Yu *et al.*, 1995; Little and Lee, 1995).

Two kinds of ribozymes have been employed widely, hairpins and hammerheads. Both catalyze sequence-specific cleavage resulting in products with a 5' hydroxyl and a 2',3'-cyclic phosphate. Hammerhead ribozymes have been used more commonly, because they impose few restrictions on the target site. Hairpin ribozymes are more stable and, consequently, function better than hammerheads at physiologic temperature and magnesium concentrations.

A number of patents have issued describing various ribozymes and methods for designing ribozymes. See, for example, U.S. Patent Nos. 5,646,031; 5,646,020; 5,639,655; 5,093,246; 4,987,071; 5,116,742; and 5,037,746, each specifically incorporated herein by reference in its entirety. However, the ability of ribozymes to provide therapeutic benefit *in vivo* has not yet been demonstrated.

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U. S. Patent No. 5,354,855 (specifically incorporated herein by reference) reports that certain ribozymes can act as endonucleases with a sequence-specificity greater than that of

known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Six basic varieties of naturally occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, 1992). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* (1992). Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No.

EP 0360257), Hampel and Tritz (1989), Hampel *et al.* (1990) and U. S. Patent 5,631,359 (specifically incorporated herein by reference). An example of the hepatitis δ virus motif is described by Perrotta and Been (1992); an example of the RNaseP motif is described by Guerrier-Takada *et al.* (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990; Saville and Collins, 1991; Collins and Olive, 1993); and an example of the Group I intron is described in U. S. Patent 4,987,071 (specifically incorporated herein by reference). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

In certain embodiments, it may be important to produce enzymatic cleaving agents that exhibit a high degree of specificity for the RNA of a desired target, such as one of the sequences disclosed herein. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target mRNA. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required, although in preferred embodiments the ribozymes are expressed from DNA or RNA vectors that are delivered to specific cells.

Small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead or the hairpin structure) may also be used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. Alternatively, catalytic RNA molecules can be expressed within cells from eukaryotic promoters (*e.g.*, Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992; Dropulic *et al.*, 1992; Weerasinghe *et al.*, 1991; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Sarver *et al.*, 1990). Those skilled in the art realize that any ribozyme can be expressed in eukaryotic cells from the appropriate DNA vector. The activity of such ribozymes can be augmented by their release from the primary transcript by a second ribozyme (Int. Pat. Appl. Publ. No. WO 93/23569, and Int. Pat. Appl. Publ. No. WO 94/02595, both hereby incorporated by reference; Ohkawa *et al.*, 1992; Taira *et al.*, 1991; and Ventura *et al.*, 1993).

Ribozymes may be added directly, or can be complexed with cationic lipids, lipid complexes, packaged within liposomes, or otherwise delivered to target cells. The RNA or RNA complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, aerosol inhalation, infusion pump or stent, with or without their incorporation in biopolymers.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595 (each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Hammerhead or hairpin ribozymes may be individually analyzed by computer folding (Jaeger *et al.*, 1989) to assess whether the ribozyme sequences fold into the appropriate secondary structure, as described herein. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 or so bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Ribozymes of the hammerhead or hairpin motif may be designed to anneal to various sites in the mRNA message, and can be chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.* (1987) and in Scaringe *et al.* (1990) and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. Average stepwise coupling yields are typically >98%. Hairpin ribozymes may be synthesized in two parts and annealed to reconstruct an active ribozyme (Chowrira and Burke, 1992). Ribozymes may be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see *e.g.*, Usman and Cedergren, 1992). Ribozymes may be purified by gel electrophoresis using general methods or by high-pressure liquid chromatography and resuspended in water.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Perrault *et al.*, 1990; Pieken *et al.*, 1991; Usman and Cedergren, 1992; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U.S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

A preferred means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I),

RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990; Gao and Huang, 1993; Lieber *et al.*, 1993; Zhou *et al.*, 1990). Ribozymes expressed from such promoters can function in mammalian cells (Kashani-Sabet *et al.*, 1992; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Yu *et al.*, 1993; L'Huillier *et al.*, 1992; Lisiewicz *et al.*, 1993). Although incorporation of the present ribozyme constructs into adeno-associated viral vectors is preferred, such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, other viral DNA vectors (such as adenovirus vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraocular, retinal, subretinal, intraperitoneal, intracerebroventricular, intrathecal delivery, and/or direct injection to one or more tissues of the brain. More detailed descriptions of ribozyme and rAAV vector delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Ribozymes and the AAV vectored-constructs of the present invention may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of one or more neural diseases, dysfunctions, cancers, and/or disorders. In this manner, other genetic targets may be defined as important mediators of the disease. These studies lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple ribozymes targeted to different genes, ribozymes coupled

with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).

4.17 ANTISENSE POLYNUCLEOTIDES AND OLIGONUCLEOTIDES

In certain embodiments, the AAV constructs of the invention will find utility in the delivery of antisense oligonucleotides and polynucleotides for inhibiting the expression of a selected mammalian mRNA in neural cells.

In the art the letters, A, G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adenosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uridine (Ura). As used in the specification and claims, compounds that are "antisense" to a particular PNA, DNA or mRNA "sense" strand are nucleotide compounds that have a nucleoside sequence that is complementary to the sense strand. It will be understood by those skilled in the art that the present invention broadly includes polynucleotides and smaller oligonucleotide compounds that are capable of binding to the selected DNA or mRNA sense strand. It will also be understood that mRNA includes not only the ribonucleotide sequences encoding a protein, but also regions including the 5'-untranslated region, the 3'-untranslated region, the 5'-cap region and the intron/exon junction regions.

The invention includes compounds which are not strictly antisense; the compounds of the invention also include those polynucleotides and oligonucleotides that may have some bases that are not complementary to bases in the sense strand provided such compounds have sufficient binding affinity for the particular DNA or mRNA for which an inhibition of expression is desired. In addition, base modifications or the use of universal bases such as inosine in the oligonucleotides of the invention are contemplated within the scope of the subject invention.

The antisense compounds may have some or all of the phosphates in the nucleotides replaced by phosphorothioates ($X=S$) or methylphosphonates ($X=CH_3$) or other C_{1-4} alkylphosphonates. The antisense compounds optionally may be further differentiated from native DNA by replacing one or both of the free hydroxy groups of the antisense molecule with C_{1-4} alkoxy groups ($R=C_{1-4}$ alkoxy). As used herein, C_{1-4} alkyl means a branched or unbranched hydrocarbon having 1 to 4 carbon-atoms.

The disclosed antisense compounds also may be substituted at the 3' and/or 5' ends by a substituted acridine derivative. As used herein, "substituted acridine," means any acridine derivative capable of intercalating nucleotide strands such as DNA. Preferred substituted acridines are 2-methoxy-6-chloro-9-pentylaminoacridine, *N*-(6-chloro-2-methoxyacridinyl)-

O-methoxydiisopropylaminophosphinyl-3-aminopropanol, and *N*-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-5-aminopentanol. Other suitable acridine derivatives are readily apparent to persons skilled in the art. Additionally, as used herein "P(O)(O) -substituted acridine" means a phosphate covalently linked to a substitute acridine.

As used herein, the term "nucleotides" includes nucleotides in which the phosphate moiety is replaced by phosphorothioate or alkylphosphonate and the nucleotides may be substituted by substituted acridines.

In one embodiment, the antisense compounds of the invention differ from native DNA by the modification of the phosphodiester backbone to extend the life of the antisense molecule. For example, the phosphates can be replaced by phosphorothioates. The ends of the molecule may also be optimally substituted by an acridine derivative that intercalates nucleotide strands of DNA. Intl. Pat. Appl. Publ. No. WO 98/13526 and U. S. Patent 5,849,902 (each specifically incorporated herein by reference in its entirety) describe a method of preparing three component chimeric antisense compositions, and discuss many of the currently available methodologies for synthesis of substituted oligonucleotides having improved antisense characteristics and/or half-life.

The reaction scheme involves ¹H-tetrazole-catalyzed coupling of phosphoramidites to give phosphate intermediates that are subsequently reacted with sulfur in 2,6-lutidine to generate phosphate compounds. Oligonucleotide compounds are prepared by treating the phosphate compounds with thiophenoxide (1:2:2 thiophenol/triethylamine/tetrahydrofuran, room temperature, 1 hr). The reaction sequence is repeated until an oligonucleotide compound of the desired length has been prepared. The compounds are cleaved from the support by treating with ammonium hydroxide at room temperature for 1 hr and then are further deprotected by heating at about 50°C overnight to yield preferred antisense compounds.

Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, *T_m*, binding energy, relative stability, and antisense compositions were selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those that are at or near the AUG translation initiation codon, and those sequences that were substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations were performed using v.4 of the OLIGO primer

analysis software (Rychlik, 1997) and the BLASTN 2.0.5 algorithm software (Altschul *et al.*, 1997).

4.18 EXEMPLARY DEFINITIONS

In accordance with the present invention, polynucleotides, nucleic acid segments, nucleic acid sequences, and the like, include, but are not limited to, DNAs (including and not limited to genomic or extragenomic DNAs), genes, peptide nucleic acids (PNAs) RNAs (including, but not limited to, rRNAs, mRNAs and tRNAs), nucleosides, and suitable nucleic acid segments either obtained from natural sources, chemically synthesized, modified, or otherwise prepared or synthesized in whole or in part by the hand of man.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and compositions similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and compositions are described herein. For purposes of the present invention, the following terms are defined below:

A, an: In accordance with long standing patent law convention, the words “a” and “an” when used in this application, including the claims, denotes “one or more”.

Expression: The combination of intracellular processes, including transcription and translation undergone by a polynucleotide such as a structural gene to synthesize the encoded peptide or polypeptide.

Promoter: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Regulatory Element: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription. Exemplary regulatory elements include, but are not limited to, enhancers, post-transcriptional elements, transcriptional control sequences, and such like.

Structural gene: A polynucleotide, such as a gene, that is expressed to produce an encoded peptide, polypeptide, protein, ribozyme, catalytic RNA molecule, or antisense molecule.

Transformation: A process of introducing an exogenous polynucleotide sequence (*e.g.*, a viral vector, a plasmid, or a recombinant DNA or RNA molecule) into a host cell or protoplast in which the exogenous polynucleotide is incorporated into at least a first chromosome or is capable of autonomous replication within the transformed host cell.

Transfection, electroporation, and “naked” nucleic acid uptake all represent examples of techniques used to transform a host cell with one or more polynucleotides.

Transformed cell: A host cell whose nucleic acid complement has been altered by the introduction of one or more exogenous polynucleotides into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell, or from the progeny or offspring of any generation of such a transformed host cell.

Vector: A nucleic acid molecule (typically comprised of DNA) capable of replication in a host cell and/or to which another nucleic acid segment can be operatively linked so as to bring about replication of the attached segment. A plasmid, cosmid, or a virus is an exemplary vector.

The terms “substantially corresponds to”, “substantially homologous”, or “substantial identity” as used herein denotes a characteristic of a nucleic acid or an amino acid sequence, wherein a selected nucleic acid or amino acid sequence has at least about 70 or about 75 percent sequence identity as compared to a selected reference nucleic acid or amino acid sequence. More typically, the selected sequence and the reference sequence will have at least about 76, 77, 78, 79, 80, 81, 82, 83, 84 or even 85 percent sequence identity, and more preferably at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 percent sequence identity. More preferably still, highly homologous sequences often share greater than at least about 96, 97, 98, or 99 percent sequence identity between the selected sequence and the reference sequence to which it was compared. The percentage of sequence identity may be calculated over the entire length of the sequences to be compared, or may be calculated by excluding small deletions or additions which total less than about 25 percent or so of the chosen reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, in the case of sequence homology of two or more polynucleotide sequences, the reference sequence will typically comprise at least about 18-25 nucleotides, more typically at least about 26 to 35 nucleotides, and even more typically at least about 40, 50, 60, 70, 80, 90, or even 100 or so nucleotides. Desirably, which highly homologous fragments are desired, the extent of percent identity between the two sequences will be at least about 80%, preferably at least about 85%, and more preferably about 90% or 95% or higher, as readily determined by one or more of the sequence comparison algorithms well-known to those of skill in the art, such as e.g., the FASTA program analysis described by Pearson and Lipman (1988).

The term “naturally occurring” as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by the hand of man in a laboratory is naturally-occurring. As used herein, laboratory strains of rodents that may have been selectively bred according to classical genetics are considered naturally occurring animals.

As used herein, a “heterologous” is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter which does not naturally occur adjacent to the referenced structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

“Transcriptional regulatory element” refers to a polynucleotide sequence that activates transcription alone or in combination with one or more other nucleic acid sequences. A transcriptional regulatory element can, for example, comprise one or more promoters, one or more response elements, one or more negative regulatory elements, and/or one or more enhancers.

As used herein, a “transcription factor recognition site” and a “transcription factor binding site” refer to a polynucleotide sequence(s) or sequence motif(s) which are identified as being sites for the sequence-specific interaction of one or more transcription factors, frequently taking the form of direct protein-DNA binding. Typically, transcription factor binding sites can be identified by DNA footprinting, gel mobility shift assays, and the like, and/or can be predicted on the basis of known consensus sequence motifs, or by other methods known to those of skill in the art.

As used herein, the term “operably linked” refers to a linkage of two or more polynucleotides or two or more nucleic acid sequences in a functional relationship. A nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. “Operably linked” means that the nucleic acid sequences being linked are typically contiguous, or substantially contiguous, and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

“Transcriptional unit” refers to a polynucleotide sequence that comprises at least a first structural gene operably linked to at least a first *cis*-acting promoter sequence and optionally linked operably to one or more other *cis*-acting nucleic acid sequences necessary for efficient transcription of the structural gene sequences, and at least a first distal regulatory element as may be required for the appropriate tissue-specific and developmental transcription of the structural gene sequence operably positioned under the control of the promoter and/or enhancer elements, as well as any additional *cis* sequences that are necessary for efficient transcription and translation (*e.g.*, polyadenylation site(s), mRNA stability controlling sequence(s), *etc.*

The term “substantially complementary,” when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an oligonucleotide sequence, is substantially complementary to all or a portion of the selected sequence, and thus will specifically bind to a portion of an mRNA encoding the selected sequence. As such, typically the sequences will be highly complementary to the mRNA “target” sequence, and will have no more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the complementary portion of the sequence. In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary to the sequence to which the oligonucleotide specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region of the mRNA and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product.

Substantially complementary oligonucleotide sequences will be greater than about 80 percent complementary (or ‘% exact-match’) to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and

even 100% exact match complementary to all or a portion of the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

5. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – IMPROVED RAAV VECTORS HAVING GENETIC MODIFICATIONS IN SPECIFIC CAPSID PROTEINS

Given advances in purification methods for rAAV2, the requirements of the individual capsid protein species in rAAV2 particle formation were reexamined in the context of designing a novel rAAV2 production system that would allow for the modification of a specific capsid protein in regions of capsid sequence overlap. Currently, highly purified and concentrated preparations of rAAV2 particles are possible from two plasmid-based production systems. These systems differ in that one system supplies the necessary adenovirus helper functions and AAV rep and cap genes from one plasmid (pDG), while the other uses two plasmids to supply these proteins (pIM45 and pXX6). These constructs are

transfected into an appropriate cell type along with a construct containing a transgene expression cassette flanked by the AAV terminal repeats (*e.g.*, pTR-UF5). This example describes an rAAV2 production system based on modifications of the triple plasmid transfection method. In this system, the expression of a specific capsid protein is restricted to one pIM45 plasmid and complemented in trans with the remaining two capsid proteins expressed from a second pIM45 plasmid. This approach maintains expression of the capsid proteins in their genomic context while providing a platform for the genetic modification of a specific capsid protein or two of the capsid proteins across their entire coding sequence. Missense mutation of the capsid proteins' start codons generated pIM45 plasmids that express a single capsid protein: pIM45-VP1, pIM45-VP2 (ACG or ATG start codon), and pIM45-VP3. Such plasmids can be complemented with plasmids expressing the remaining 2 capsid proteins (pIM45-VP2,3, pIM45-VP1,3, and pIM45-VP1,2 (ACG or ATG start codon), respectively) in order to produce viable rAAV2 vectors. Using the system's plasmid components individually, a reevaluation of capsid protein requirements for the production of rAAV2 particles revealed that viable rAAV2-like particles are produced as long as the VP3 protein is present (VP1+2+3, VP1+3, VP2+3, and VP3 only). Focusing on large peptide insertions in the VP1 and VP2 proteins without altering the critical VP3 protein, the utility of this system is demonstrated through the production of viable rAAV2 particles containing 8-, 15-, and 29-kDa proteins inserted immediately following amino acid 138 in both VP1 and VP2 proteins or in VP2 protein alone. Finally, rAAV2-like particles can be produced with altered capsid protein stoichiometry if VP2 is significantly over expressed.

5.1.1 CONSTRUCTION OF RAAV2 CAPSID MUTANT PLASMIDS THAT EXPRESS TWO CAPSID PROTEINS

To isolate the expression of a specific capsid protein to one pIM45 plasmid and the remaining two capsid proteins to a second pIM45 plasmid, missense mutation of the AAV2 cap ORF start codons was employed as previously described. Using site-directed mutagenesis of a pIM45 template, the VP1 start codon was mutated to leucine to generate the construct, pIM45-VP2,3, the VP2 start codon to alanine to generate the construct, pIM45-VP1,3, and the VP3 start codon to leucine to generate the construct, pIM45-VP1,2 (FIG. 1A). Western blotting analysis of capsid protein expression in whole cell lysates 48 hours post transfection of 293 cells with these plasmids in the presence of Ad5 (MOI=10) was carried out using the B1 antibody which recognizes all three capsid proteins (FIG. 1A). As previously reported, the expression of VP1 and VP2 could be eliminated by missense

mutation of their start codons (FIG. 1A, lanes 2 and 3), and, in contrast, mutation of the VP3 start codon resulted in expression of a smaller VP3-like fragment (VP3a) (FIG. 1A, lane 4). Since this construct did not eliminate all VP3-like proteins it was renamed, pIM45-M203L. In the baculovirus study of AAV particle assembly, it was suggested that mutation of the VP3 start codon allows translational initiation to occur downstream at the next available ATG codon with correct Kozak sequences. While no additional ATG codons are found between the VP1 start codon and the start of VP3, an examination of the VP3 capsid revealed that nine additional ATG codons are present (amino acid positions 211, 235, 371, 402, 434, 523, 558, 604, and 634). Of these methionines, only those at amino acid position 211, 235, 523, 558, and 604 are in a context that is predicted favorable by Kozak. Since the VP3a fragment is slightly smaller than wildtype VP3, the contribution of continued read through translational initiation to the appearance of the VP3a fragment was examined by mutating the next two available ATG codons (M211 and M235) on a pIM45-M203L template yielding the plasmids, pIM45-M203L, pIM45-M203,211L and pIM45-M203,211,235L (FIG. 2A). Western blotting analysis of capsid protein expression in whole cell lysates 48 hours post transfection of 293 cells in the presence of Ad5 (MOI=10) revealed that translational initiation could occur at both these ATG codons. FIG. 1B (lane 2) again demonstrates the formation of VP3a following the mutation M203L. Combined mutation of M203 and M211 allowed less robust expression of a second still shorter VP3-like fragment (VP3b, FIG. 1B, lane 3). Subsequent mutation of M235 in the pIM45-M203,211L background led to disappearance of this VP3b fragment generating pIM45-VP1,2 (FIG. 1B, lane 4). Collectively, while missense mutagenesis of the VP1 start codon does not alter the sequence of the VP2 and VP3 protein expressed (pIM45-VP2,3, M1L), mutation of the VP2 start codon results in one point mutation in the expressed VP1 protein (pIM45-VP1,3, T138A), and elimination of all VP3-like proteins results in three mutations in the remaining VP1 and VP2 proteins (pIM45-VP1,2, M203,211,235L).

An alternative method has been reported for eliminating VP3 expression that limits mutation of remaining capsid sequences to one point mutation in the VP2 start codon. Changing the VP2 start codon from ACG to ATG results in loss of VP3 expression (pIM45-VP1,2A) with one point mutation in both the VP1 and VP2 proteins (T138M). Presumably, this stronger VP2 start codon prevents efficient translational initiation at the downstream VP3 start codon. The VP2 start codon was mutated to ATG on a pIM45 template (pIM45-VP1,2A (FIG. 1C)) as an alternative means of eliminating VP3 protein (while maximizing VP2 expression). As expected, Western blotting analysis of capsid protein expression in

whole cell lysates 48 hr post transfection of 293 cells in the presence of Ad5 (MOI=10) with pIM45-VP1,2A showed normal levels of VP1 protein produced, with significantly increased expression of VP2 protein (FIG. 1C, lane 2).

5.1.2 CONSTRUCTION OF rAAV2 CAPSID PLASMID MUTANTS THAT EXPRESS A SINGLE CAPSID PROTEIN

To complete the complementary pIM45 capsid groups, pIM45 plasmids that express a single capsid protein were generated next. Employing the same missense mutations described above on templates that now only express two capsid proteins, the plasmids, pIM45-VP1, pIM45-VP2, pIM45-VP2A, and pIM45-VP3 (FIG. 2) were also generated. pIM45-VP1, has the VP2 start codon mutated to alanine and M203, M211, and M235 mutated to L in the expressed VP1 protein. pIM45-VP2 has the VP1 start codon mutated to leucine and M203, M211, and M235 mutated to L. The expressed VP2 protein contains only M203, M211, and M235 mutations. pIM45-VP3 has the VP1 start codon mutated to leucine and the VP2 start codon mutated to alanine. Like all VP3 protein in these complementary groups, the VP3 coding sequence is not mutated. Finally, pIM45-VP2A has the VP1 start codon mutated to leucine and the VP2 start codon mutated to methionine resulting in the single T138M modification of the VP2 protein being expressed. Western blotting analysis of capsid protein expression in whole cell lysates 48 hr post transfection of 293 cells with pIM45-VP1, pIM45-VP2, pIM45-VP2A, and pIM45-VP3 in the presence of Ad5 (MOI=10) demonstrated that a single capsid protein could be expressed from the pIM45 cap ORF (FIG. 2) and completed the catalogue of plasmids required of a system for further genetic manipulation of a specific capsid protein across its entire coding sequence.

5.1.3 THE VP3 N-TERMINAL M203 AND M211 ARE CRITICAL FOR AAV PARTICLE FORMATION

As control experiments for the production of AAV particles from the complementary groups of single and double capsid expressing pIM45 plasmids, particle production was examined from the individual plasmids described. Since VP3 protein makes up the bulk of the particle, and mutagenesis studies have indicated that the N-terminal region of VP3 is important for AAV particle formation, the effects of the three mutations required to eliminate VP3 expression (M203,211,235L) were investigated on the recovery of rAAV particles following standard production and purification protocols. The plasmids pIM45-M203L, pIM45-M211L, pIM45-M235L, and pIM45M-203,211,235L were cotransfected separately

with pTR-UF5 and pXX6 in a 1:1:8 molar ratio in 293 cells and 72 hrs later the cells were harvested and particles were purified as previously reported. Western blotting of capsid protein expression and dot blot analysis of genome containing particles was carried out on the mutant virus preparations (FIG. 3A). No particles were recovered from pIM45-M203L (lane 2) indicating that the combination of VP1, VP2, and VP3a does not able form a stable AAV particle. Equally important in the formation of the particle is M211 (lane 3), as this mutation also prevented particle recovery. Whether it is the M211L in VP1, VP2, or VP3 that leads to this defective phenotype is unclear. This issue is addressed *infra* when pIM45-VP1,2 is complemented with pIM45-VP3 to produce AAV particles (Fig 4 #5). Finally, particles were obtained from pIM45-M235L (Fig 3A, lane 4) that package DNA efficiently.

5.1.4 AAV-LIKE PARTICLES CAN BE PRODUCED THAT LACK VP1 OR VP2 PROTEIN

While the effect of mutating the individual capsid start codons on the formation of infectious AAV particles has been reported, given the improvements in AAV2 production and purification methods, control experiments were performed to reexamine the role of each capsid protein in the formation of the AAV2 particle capable of binding heparin. First examined were the effects of the elimination of one capsid protein on AAV2 particle recovery. pIM45-VP2,3, pIM45-VP1,3, pIM45-VP1,2, and pIM45-VP1,2A were transfected separately into 293 cells with pTR-UF5 and pXX6 in a 1:1:8 molar ratio and 72 hrs later the cells were harvested and particles were purified as previously reported. Western blotting, A20 ELISA, and dot blot analysis of these virus preparations were carried out (FIG. 3B) and, in agreement with previous reports, the elimination of the VP1 protein (pIM45-VP2,3) resulted in the production of an AAV-like particle that packaged genomes efficiently (lane 4). Surprisingly, in contrast with the initial report mapping the capsid start codons, transfection of the pIM45-VP1,3 plasmid resulted in the purification of an AAV-like particle capable of packaging genomes efficiently composed of only VP1 and VP3 (lane 3) that had only a modest decrease in infectivity compared to particles containing all three capsid proteins (two-fold decrease). Finally, regardless if VP2 is overexpressed, particles composed of only VP1 and VP2 were not recovered (lane 2).

5.1.5 AAV-LIKE PARTICLES CAN BE PRODUCED COMPOSED ONLY OF VP3 CAPSID PROTEINS

As with the pIM45 plasmids that express two capsid proteins, the ability of a single capsid protein to form an AAV-like particle was tested. pIM45-VP1, pIM45-VP2, pIM45-

VP2A, and pIM45-VP3 were transfected separately into 293 cells with pTR-UF5 and pXX6 in a 1:1:8 molar ratio and harvested cells 72 hrs later and purified particles as previously described. Western blotting of capsid proteins, A20 ELISA, and dot blot analysis of virus preparations were carried out with no detectable AAV-like particles obtained from pIM45-VP1, pIM45-VP2, or pIM45-VP2A (FIG. 3C, lanes 2 and 3). Interestingly, like a recent insertional mutagenesis study of the cap ORF, an AAV-like particle composed exclusively of VP3 protein was purified (lane 3). Like the VP2,3 AAV-like particle, this particle had a significantly lower infectious phenotype.

5.1.6 RAAV PARTICLES WITH ALL THREE CAPSID PROTEINS CAN BE PRODUCED FROM CAPSID COMPLEMENTATION GROUPS

Given the results of the control experiments, the ability to recover rAAV2 particles containing all three capsid proteins following transfection of two complementary pIM45 plasmids was tested (FIG. 4). To control for twice the Rep expression resulting from two pIM45 plasmids, an additional plasmid was constructed, pIM45-VP0, that expresses no capsid proteins as a result of 5 point mutations (M1L, T138A, M203,211,235L). Complementary group VP0 (FIG. 4, #1) includes pIM45 and pIM45-VP0, group VP1 includes pIM45-VP1 and pIM45-VP2,3 (FIG. 4, #2), group VP2 includes pIM45-VP2 and pIM45-VP1,3 (FIG. 4, #3), group VP2A includes pIM45-VP2A and pIM45-VP1,3 (FIG. 4, #4), and group VP3 includes pIM45-VP3 and pIM45-VP1,2 (FIG. 4, #5). Western blotting of capsid proteins, A20 ELISA, and dot blot analysis of virus preparations were carried out following transfection of the individual groups into 293 cells with pTR-UF5 and pXX6 in a 1:1:8 molar ratio. 72 hrs post transfection the cells were harvested and particles were purified as previously described. Infectious rAAV particles containing all three capsid proteins with similar yields were recovered (FIG. 5A). Interestingly, the VP2A group resulted in the formation of particles with an apparent alteration of capsid protein stoichiometry and lower infectivity compared to other groups (lane 4). The characteristics of this group suggest that this preparation may contain a single unique particle that is defective *per se* or alternatively two particles may be assembled containing all three capsid proteins at normal levels and a defective interfering particle composed of VP2 and VP3 proteins with altered stoichiometry. Cotransfection of the pIM45-VP2A and pIM45-VP3 plasmid should yield a particle with an altered VP2:VP3 ratio if such a defective interfering particle contributes to the low titer of this group. Indeed, an AAV-like particle with an

overrepresentation of VP2 protein was purified that resembled the VP2,3 and VP3 only particles with respect to infectivity (FIG. 5B, lane 4).

5.1.7 PRODUCTION OF AAV PARTICLES WITH INSERTIONS IN THE VP1/VP2 OVERLAP REGION

Since the VP1/VP2 overlap region has been shown to be on the surface of the particle and flexible in the acceptance of targeting epitopes, the ability of this region to accept larger insertions was examined. Presumably, large insertions in the VP3 protein would decrease ones success in obtaining a particle due to steric hindrances in assembling the 60 modified capsid subunits. Sixty ligands were considered excessive when inserting large molecules into the AAV particle, so the strategy employed was to focus larger insertions to VP1 and/or VP2 proteins. Large insertions in both VP1 and VP2 protein immediately after amino acid 138 may have less steric constraints but may produce particles with defective trafficking due to the juxtaposition of a large insertion to the putative phospholipase motif in VP1 protein. Also, since VP1, essentially an N-terminal fusion of 137 amino acids to VP2, and a CD 34 sc antibody VP2 protein fusion are readily incorporated into an AAV particle, insertion of large epitopes only at the N-terminus of VP2 may have advantages. Notably, genetic modification of the VP2 protein exclusively has not been accomplished from within a pIM45 based AAV production scheme. To address the ability to insert large peptide sequences in the VP1/VP2 overlap region of the cap ORF, directional cloning sites were generated immediately after amino acid 138 in plasmids that express VP1 and VP2 or VP2 only (FIG. 6A). The choice of *EagI/MluI* restriction sites resulted in two amino acids insertions on either side of further inserted sequence (RP/RT). pIM45-VP1,2A and pIM45-VP2A were chosen as templates for engineering *EagI/MluI* restriction sites immediately after amino acid 138 in VP1 and VP2 or in VP2 only (pIM45VP1,2AEM138, pIM45-VP2AEM138, FIG. 6A). Templates with VP2 protein over expression were used to help ensure that genetically-modified VP2 would be present at sufficient levels for assembly. The pIM45-VP1,2AEM138 plasmid was complemented with pIM45-VP3, while the pIM45-VP2AEM138 plasmid was complemented with pIM45-VP1,3 for the production of rAAV particles carrying insertion sequences. Insertion of the coding sequence for leptin and GFP was in these plasmids generated pIM45-VP1,2Alep, pIM45-VP2Alep, pIM45-VP1,2AGFP, and pIM45-VP2AGFP. These plasmids and their complements were transfected with in 293 cells in the presence of pTR-UF5 (leptin-insertions) or pTR-dsRFP (GFP) and pXX6 in a 1:1:8 molar ratio. 72 hrs post transfection the cells were harvested and virus particles were purified as previously described. Western

blotting, A20 ELISA, and dot blot analysis were carried out on the virus preparations (FIG. 6B and FIG. 6C). For the leptin-inserted virus preparations successful insertion in both VP1 and VP2 or VP2 only was possible in the purified particle, but GFP-insertion in the purified particle was only possible in the VP1 protein (VP2-GFP was excluded in both cases).

5.1.8 DISCUSSION

This example increases the flexibility in probing the surface of the particle by isolating the expression of a given capsid protein to a separate plasmid. Such an approach allows for manipulation of this capsid protein only within the produced particle and allows for retesting regions of capsid overlap for the acceptance of sequence modification. Alternatively, the system also allows for the modification of only two of the capsid proteins while leaving the third protein unmodified. Using the missense mutation of capsid start codons to generate all required plasmids, characterization of the catalogue of plasmids required for this system yielded interesting results concerning the role of each capsid protein in the assembly of AAV-like particles.

Elimination of VP3-like fragments illustrates importance of VP3 N terminus, as particles with these mutations in the VP1 and VP2 proteins were recovered following complementation of pIM45-VP1,2 with pIM45-VP3. Evidence that ability to modify individual capsid proteins in regions of overlap may allow production of particles that were defective for production when mutations are in all three proteins. Increases the flexibility in manipulation of the particle for targeting purposes. Recently, isolation of the expression of a C-terminal modified VP3 separately allowed for modification of the c-terminus of VP3 with his tag and production of viable recombinant virus follow nickel chromatography. Like the study involving the VP3-6xHis tag where the modified capsid protein was isolated and VP1 and VP2 did not carry the insertion, lethal mutations in the overlapping N-terminus region of VP3 (M203,211) resulted in particles from complementation group 3 when these mutations were only in VP1 and VP2 with normal VP3.

The present system allows for complementation and recovery of rAAV2 particles with all capsid proteins present. Since it allows for the genetic modification of only one or two of the capsid proteins, it can also be used for studies of previously reported lethal mutations in overlapping capsid sequences to see if mutations at the same positions in fewer capsid proteins rescue the position for particle manipulation. Important genetic modification would include insertion of genetic sequence for retargeting the virus, purification of the virus,

monitoring of the virus particle following infection, or presentation of immunogenic epitopes on the surface of the virus particle.

Insertions of large peptides (leptin and GFP) into the overlapping region of VP1 and VP2 resulted in the purification of virus like particles carrying these insertions. This required preliminary isolation of the expression of VP1 and VP2 (pIM45-VP1,2A) or VP2 only (pIM45-VP2A) to a separate plasmid followed by insertion of peptide sequences after amino acid 138 allowed for the production of peptide inserted AAV-like particles following complementation with pIM45-VP3 or pIM45-VP1,3. This example is the first report of the purification of an AAV-like particle containing a mutation in the VP2 protein exclusively. Estimated similar stoichiometry of capsid proteins in particle. Retain ability to package genomes, bind A20, and are infectious as they retain native tropism due to intact heparin binding motif. VP2 overexpression may have ensured the inclusion of modified VP2 protein large insertions with VP2 acg start codon produced significantly less modified VP2 proteins.

5.2 EXAMPLE 2 – HEPARIN SULFATE BINDING MOTIF IN AAV2 CAPSID PROTEINS REQUIRED FOR NATIVE TROPISM

In this example, charged-to-alanine substitution mutants were made to analyze the effects of single and combinatorial mutations in the capsid gene. New point mutants that result in assembly, packaging, and receptor binding deficiencies have been discovered. Importantly, five amino acids, arginines 484, 487, 585, and 588, and one lysine at position 532 have been identified that appear to mediate the natural affinity of AAV for HSPG. Those observations contribute to the current map of the AAV capsid and provide a reagent for the discovery of novel, heparin independent targeting ligands.

5.2.1 MATERIALS AND METHODS

5.2.1.1 PLASMIDS

Plasmid pIM45 (previously called pIM29-45) contains the Rep and Cap coding sequences from AAV with expression controlled by their natural promoters (McCarty *et al.*, 1991). It was used as the parent template for construction of all the AAV2 mutant vectors.

Plasmid pXX6 supplies the adenovirus helper gene products in *trans* to allow rAAV production in an adenovirus free environment (Xiao *et al.*, 1998).

Plasmid pTR2-UF5 supplies the recombinant AAV DNA to be packaged. It contains a cytomegalovirus promoter driving expression of a green fluorescent protein (GFP) reporter gene flanked by AAV2 terminal repeats (Klein *et al.*, 1998). Plasmid pTR5-UF11 was

constructed using an expression cassette consisting of a strong constitutive CBA promoter (Xu *et al.*, 2001), GFP reporter gene (Zolotukhin *et al.*, 1996), woodchuck hepatitis virus posttranscriptional regulatory element WPRE (Donello *et al.*, 1998) and bovine growth hormone gene polyadenylation signal. The cassette was assembled using standard molecular biology techniques and substituted for the *lacZ* cassette in the plasmid backbone pAAV5Rn*lacZ* containing AAV5 terminal repeats (Chiorini *et al.*, 1999).

Plasmids pXYZ1, pXYZ5 contain the AAV1 and AAV5 Cap coding sequences, respectively, in addition to AAV2 Rep coding sequence with an ACG start codon under control of the AAV2 p5 promoter (Zolotukhin *et al.*, 2002). Plasmid pAAV5-2 contains the AAV5 nucleotides 260 to 4448 without terminal repeats (Chiorini *et al.*, 1999).

5.2.1.2 CONSTRUCTION OF MUTANT CAPSID PLASMIDS

Quickchange site directed mutagenesis (Stratagene) was performed on plasmid pIM45 as per the manufacturer's instructions. For each AAV2 mutant, two complementary PCR primers that contained alanine or lysine substitutions in addition to a silent change for restriction endonuclease screening purposes were used to introduce changes into pIM45. For construction of AAV5-HS, pAAV5-2 was used as the parental template. Sequences for the oligonucleotides used are available upon request. PCR products were digested with DpnI to remove methylated template DNA, phenol:chloroform:isoamyl (25:24:1) extracted, ethanol precipitated, and transformed into electrocompetent JM109 cells. Miniprep DNA was extracted from overnight LB/amp cultures and screened with the appropriate restriction enzyme. All mutants were sequenced prior to use. Transfection quality plasmid DNA was produced by standard alkaline lysis method of a 1-liter TB culture followed by PEG precipitation and cesium chloride gradient purification.

5.2.1.3 CELL CULTURE

Human embryonic kidney 293's and cervical carcinoma HeLa C12's, a gift from Dr. Phil Johnson (Clark *et al.*, 1996) were grown in Dulbecco Modified Eagle Medium (Gibco-BRL) supplemented with 100U/ml penicillin, 100U/ml streptomycin, 10% bovine calf serum, sodium pyruvate and L-glutamine. Cells were incubated at 37°C in a 5% CO₂ atmosphere.

5.2.1.4 PRODUCTION OF RAAV2 PARTICLES

To produce AAV2 virions, low passage 293's were seeded so that they were approximately 75% confluent at transfection time. A triple plasmid transfection protocol

(Xiao *et al.*, 1998) was followed that included pIM45 to supply Rep and mutated capsid genes, pTR2-UF5 (Klein *et al.*, 1998) to supply recombinant DNA with AAV2 terminal repeats and a CMV driven GFP reporter gene, and pXX6 (Xiao *et al.*, 1998) to supply the adenovirus helper functions in *trans*. A total of 60 µg of plasmid DNA in a 1:1:1 molar ratio was transfected by lipofectamine (Invitrogen).

To produce pseudotyped rAAV1 and rAAV5 particles, a total of 60 µg of pXYZ1 or pXYZ5 (Zolotukhin *et al.*, 2002) was co-transfected with pTR2-UF5 plasmid DNA in a 1:1 molar ratio as above. To produce rAAV5 and rAAV5-HS virions a total of 60 µg of pAAV5 or pAAV5-HS was co-transfected with pTR5-UF11.

Purification of rAAV has been described previously (Zolotukhin *et al.*, 1999; Zolotukhin *et al.*, 2002). Briefly, 72 hr after transfection, cells were harvested and the pellets were resuspended in lysis buffer (.15M NaCl, 50 mM Tris-Cl pH=8.5). Virus was released by three cycles of freezing and thawing. Benzonase (Sigma) was added to the cell lysate to a final concentration of 140 U/ml and incubated at 37°C for 30 min. Cell debris was pelleted by centrifugation at 3,700× g for 30 min and the supernatant was loaded onto a 15%-25%-40%-60% iodixanol (5,5'[2-hydroxy-1,3-propanediyl]bis(acetyl-amino)] bis[N,N'-bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide)] step gradient (Nycomed). The 40% fraction was collected after centrifugation at 69,000× g for 1 hr and stored at -80°C until further use.

5.2.1.5 VIRUS TITER DETERMINATION

To determine the concentration of intact capsid particles, the A20 ELIZA (American Research Bioproducts) was used. The A20 antibody detects intact, fully assembled particles, both full and empty (Wistuba *et al.*, 1995). Iodixanol purified stocks were serially diluted and processed by the manufacturer's recommended protocol. Only readings within the linear range of the kit standard were used.

To determine the concentration of DNA-containing particles, real-time (RT)-PCR™ was performed using a Perkin Elmer-Applied Biosystems (Foster City, CA) Prism 7700 sequence detector system. Equal volumes of iodixanol purified virus stocks were treated with 600 U/ml benzonase in 50 mM Tris-CL pH=7.5, 10 mM MgCl₂, 10 mM CaCl₂ at 37°C for 30 min. 280 U/ml proteinase-K was added to reactions adjusted to 10 mM EDTA and 5% SDS, and then incubated at 37°C for 30 min. Reactions were extracted with phenol/chloroform/isoamyl-alcohol (25:24:1) and undigested DNA was precipitated

overnight with ethanol and glycogen carrier. Precipitated DNA pellets were resuspended in 100 μ l of water. Five μ l was used for RT-PCR[™] analysis in a reaction mixture that included 900 nM each of GFP forward and reverse primers, 250 nM Taqman probe, 1X Taqman universal PCR master mix in a total volume of 50 μ l. Cycling parameters were 1 cycle each of 50°C, 5 mins, and 95°C, 10 mins, followed by 40 cycles of 95°C, 15 sec and 60°C, 1 min. Only values within the linear portion of a standard curve having a coefficient of linearity greater than .98 were accepted. The average RT-PCR[™] titer was calculated from virus preparations assayed three times.

To determine the infectious titer of the wt and mutant virus stocks, a green cell assay (GCA) was performed essentially as previously described (Zolotukhin *et al.*, 1999). Briefly, HeLa C12 cells were seeded in a 96 well plate so that they were approximately 75% confluent at infection time. Cells were infected with 10-fold serial dilutions of iodixanol purified mutant viruses and Ad5 at a constant multiplicity of infection (MOI) = 10. Cells were incubated at 37°C in a 5% CO₂ atmosphere for 24 hrs and examined by fluorescence microscopy. The average GCA titer was calculated by averaging the number of green cells counted in individual wells from two or three virus preparations assayed three times. Particle to infectivity ratios were calculated by dividing the average RT-PCR[™] titer by the average GCA titer. In some figures, this number was expressed as a log₁₀ value with rAAV2 arbitrarily set to one.

5.2.1.6 IN VITRO HEPARIN BINDING ASSAY

Bio-Rad microspin columns were treated with silicon dioxide to minimize non-specific binding of the virus to the column wall. A 500 μ p heparin-agarose (Sigma H-6508) gravity column was prepared by washing with 3 column volumes each of 1X TD (137 mM NaCl, 15 mM KCl, 10 mM Na₂PO₄, 5 mM MgCl₂, 2 mM KH₂PO₄, pH=7.4), 1X TD + 2M NaCl and 1X TD. Approximately equal numbers of virus particles were added to 1X TD to a final volume of 600 μ l and loaded onto the column. The column was washed with 7 column volumes of 1X TD. Bound virus was eluted with 1X TD + 2M NaCl. The entire volume of the flow through, wash, and eluate fractions were pooled separately, denatured by boiling in SDS, and slot blotted onto nitrocellulose for immunoblot analysis. The membrane (Osmonics) was blocked in PBS/0.05% Tween-20 + 5% dry milk, and incubated with B1 antibody (Wistuba *et al.*, 1997) at a 1:3000 dilution for 18 hrs at 4°C. Anti-mouse IgG-horse

radish peroxidase was used to detect bands by enhanced chemiluminescence (Amersham-Pharmacia).

5.2.1.7 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

HeLa C12 cells were seeded in 6 well plates so that they were approximately 75% confluent at infection time. Cells were infected with an rAAV MOI=500 based on the genomic titer as determined by DNA dot blot assay (Zolotukhin *et al.*, 1999). Adenovirus type-5 was used at an MOI=10 plaque forming units (pfu). Twenty-four hours postinfection, cells were washed, trypsinized, and fixed in 2% paraformaldehyde. FACS analysis for GFP expression was done in the ICBR Flow Cytometry lab of the University of Florida on a Becton-Dickinson FACScan.

5.2.1.8 CELL ATTACHMENT ASSAY

10^6 HeLa C12 cells were infected with rAAV2 at a genome containing particle MOI = 100 or R585A/R588A at an MOI = 1000 as determined by RT-PCR[™]. Cells were incubated at 37°C in a 5% CO₂ atmosphere until harvesting. At indicated time points, the infection media was removed and saved and the cells were washed four times with PBS before being scraped. Low molecular weight DNA from the infection media and the cell pellet was extracted by the Hirt procedure (Hirt, 1967). DNA pellets were resuspended in 0.2M NaOH, incubated at 37°C for 20 mins, and slot blotted onto nitrocellulose. DNA was UV cross-linked to the nitrocellulose and probed at 65°C for 18 hrs with [α -³²P]-dATP labeled GFP probe in hybridization buffer (7% SDS, 10 mM EDTA and .5M Na₂HPO₄). Membranes were washed twice in 2X SSC/0.1%SDS, 0.2XSSC/0.1%SDS, 0.1X SSC/0.1%SDS, and rinsed with water. The membranes were then exposed to film and quantitated using a BAS-1000 phosphor imager (Fuji).

5.2.2 RESULTS

5.2.2.1 SELECTION AND GENERATION OF AAV MUTANTS

A considerable body of information regarding the determinants of HS-protein interactions suggests that their association is driven mainly by electrostatic attraction between acidic sulfate groups on the polysaccharide and basic R-groups on amino acids in the target protein (Hermens *et al.*, 1999; Hileman *et al.*, 1998). It was hypothesized that similar electrostatic interactions would govern HSPG-AAV2 association. In order to evaluate the role of particular amino acids in receptor binding, a panel of mutants was generated by site

directed mutagenesis of selected residues. The selection was confined primarily to basic amino acids (His, Lys, Arg) in VP3 as AAV-like particles composed only of VP3 proteins have been purified by heparin affinity chromatography. Any basic amino acid substitution mutant that previously had demonstrated capsid instability or efficient purification by heparin affinity chromatography (Wu *et al.*, 2000) was excluded from the pool of mutants.

Seven AAV serotypes have been reported (Bantel Schaal and zur Hausen, 1984; Gao *et al.*, 2002; Hoggan *et al.*, 1996; Parks *et al.*, 1967; Rutledge *et al.*, 1998). Several groups have shown that rAAV2 and rAAV3 bind efficiently to heparin sulfate (Rabinowitz *et al.*, 2002; Shi *et al.*, 2001; Wu *et al.*, 2000). A single report concerning rAAV1 suggests that it binds with low affinity, if at all, to heparin (Rabinowitz *et al.*, 2002). In contrast, rAAV4 and rAAV5 do not bind heparin and instead recognize 2,3 O-linked and 2,6 N-linked sialic acid moieties (Kaludov *et al.*, 2001). Indeed, this may account for their different cellular tropisms. It was reasoned that residues conserved among all five serotypes were probably not participating directly in receptor discrimination and binding and were excluded from further consideration. Additionally, a number of charge to alanine substitution mutants in the AAV capsid had been identified, and these had been characterized for their ability to bind heparin sulfate columns (Wu *et al.*, 2000) and amino acid positions that did not affect heparin binding or had been shown to be assembly mutants were excluded from further study. Using a Clustal W algorithm, a sequence alignment of capsid proteins from serotypes 1-5 was generated, and 9 basic residues in AAV2 that were conserved in AAV3 and/or AAV1 but were uncharged or acidic in AAV4 and AAV5 were identified that had not previously been tested for heparin-agarose binding (Table 4). In addition to these 9 amino acids, Wu *et al.* (2000) described a virus deficient for heparin binding with alanine substitution mutations at positions 585, 587, and 588. Finally, during the course of these studies, the atomic structure of AAV2 was solved (Xie *et al.*, 2002) and suggested that residues 484, 513, and 532 might participate in a heparin-binding pocket as they were located close to residues 585, 587, and 588. These six extra residues were also included to complete the mutant panel (Table 4).

TABLE 4
RESIDUES CHOSEN FOR MUTAGENESIS

VP Residue ^a	AAV Serotype ^b				
	2	3	1	4	5
358	H	H	H	Q	T

447	R	R	R	S	S
459	R	R	D	T	G
484	R	R	R	K	R
487	R	R	R	G	G
509	H	H	H	T	E
513	R	R	R	R	A
526	H	H	H	A	N
527	K	K	K	G	N
532	K	K	K	K	N
544	K	K	K	P	S
566	R	R	K	A	Q
585	R	S	S	S	S
587	N	N	S	S	T
588	R	T	T	N	T

^a Residues selected for mutagenesis were generated by a sequence alignment of the VP1 capsid protein from each serotype using the Clustal W algorithm (Vector NTi 5.2, Informax).

^b Amino acids are represented by their one letter abbreviation. Blue letters represent positively charged, basic amino acids. Red letters represent any other amino acid.

5.2.2.2 MUTANT VIRUS PRODUCTION AND PHYSICAL CHARACTERIZATION

A series of single and combinatorial capsid mutants were generated from the pool of candidate residues in the AAV2 capsid gene (Table 4). To designate the mutant viruses, the number of the mutated amino acid based on its position in VP1 was used. Iodixanol purified virus stocks were checked by western blot using the monoclonal antibody B1. The B1 antibody recognizes a linear epitope in the extreme carboxyl terminus of all three VP proteins from AAV serotypes 1, 2, 3 and 5 (Rabinowitz *et al.*, 2002; Wobus *et al.*, 2000). With the exception of H358A, capsid proteins were detected in all virus stocks (FIG. 7). To confirm that assembled capsids, rather than subunits or assembly intermediates, had been purified, the particle concentration was measured with an A20 antibody ELISA (Table 5). The A20 antibody recognizes a structural epitope that is found only on assembled capsids with or without packaged DNA (Grimm *et al.*, 1998). Although there was some variability between stocks due to different transfection efficiencies and purification recoveries, only the H358A mutant was negative by A20 ELISA assay. Excluding H358A, a particle concentration range was determined that spanned 1.5 logs and correlated reasonably well with the B1 antibody

results (FIG. 7; Table 5). Several possibilities may account for this range of particle titers, including that capsid subunits containing these mutations (i) form intact particles inefficiently, (ii) are unstable during purification or (iii) formed a particle with a partially disrupted A20 epitope. Since none of these mutations fell within the antigenic regions that have been mapped for A20 (Wobus *et al.*, 2000), these results suggested that the A20 epitope had probably not been modified but rather the stability or assembly of some of the mutants was altered so that fewer particles were recovered after iodixanol centrifugation (FIG. 7; Table 5).

TABLE 5
TITERS AND HEPARIN BINDING PROPERTIES OF MUTANTS

Mutant virus ^a	Particle titer ^b		Infectious titer ^c	Particle to infectivity ^d	Heparin binding ^e	Empty/Full ^g
	A20/ml	Genome/ml	(IU/ml)			
rAAV2 (WT)	1.5×10^{12}	4.6×10^{11}	1.8×10^{10}	25	+	3.4
H3558A	$<1.0 \times 10^8$	$<1.0 \times 10^6$	$<1.0 \times 10^4$	N/D ^f	N/D	N/D
R447A	1.2×10^{12}	3.4×10^{10}	1.3×10^9	25	+	35.9
R459A	9.1×10^{10}	7.2×10^8	$<1.0 \times 10^4$	>72500	+	126.3
R484A	1.5×10^{11}	3.0×10^{10}	$<1.0 \times 10^4$	>2976667	+/-	5.1
R487A	5.4×10^{11}	2.2×10^{11}	2.3×10^8	954	+/-	2.5
H509A	4.6×10^{10}	2.3×10^9	6.9×10^5	3285	+	20.3
R513A	2.9×10^{11}	1.7×10^{10}	1.6×10^8	106	+	17.9
K532A	1.1×10^{11}	3.6×10^{10}	$<1.0 \times 10^4$	>3633333	+/-	3.0
K544A	2.0×10^{11}	1.7×10^{10}	8.3×10^8	20	+	11.9
R566A	5.1×10^{11}	1.6×10^{10}	7.4×10^8	21	+	32.6
R585A	5.0×10^{11}	4.8×10^{10}	1.7×10^7	2812	-	1.4
R587A	4.4×10^{11}	1.3×10^{10}	1.7×10^7	165	+	34.7
R588A	2.4×10^{11}	5.6×10^{10}	3.0×10^6	18521	-	4.2
H526A, K527A	1.4×10^{11}	8.2×10^{10}	5.5×10^7	1489	+	1.8
R585A, R588A	1.2×10^{12}	9.2×10^{11}	1.9×10^7	48421	-	1.2
R585K	1.3×10^{12}	3.7×10^{10}	4.0×10^8	92	+	35.4
R585K, R588K	1.4×10^{12}	3.9×10^{10}	8.9×10^7	436	+	34.9
AAV1	N/D	3.7×10^{10}	1.1×10^9	37	+/-	N/D
AAV5	N/D	3.4×10^{10}	3.2×10^6	10692	-	N/D

Mutant virus ^a	Particle titer ^b		Infectious titer ^c	Particle to infectivity ^d	Heparin binding ^e	Empty/ Full ^g
	A20/ml	Genome/ml	(IU/ml)			
AAV5-HS	N/D	8.0×10^8	$<1.0 \times 10^4$	>80000	+	N/D

^a Two letters flanking a number designate each mutant. The first letter is the one letter abbreviation for the wild type amino acid followed by its numerical position in VP1 followed by the one letter abbreviation for the amino acid to which it was mutated.

^b A20 particle titers were determined as described using the A20 ELISA assay. Genomic titers were determined by RT-PCRTM.

^c Infectious titers were determined by green cell assay as described by counting GFP fluorescent cells.

^d Particle-to-infectivity ratio was calculated by dividing the average genomic titer as determined by RT-PCRTM by the average green cell assay titer.

^e Determined by heparin-agarose binding assay. +, >95% virus recovered in the eluate; +/-, >50 recovered in the eluate; -, <5% of virus recovered in the eluate.

^f N/D, not determined.

^g Empty-to-full ratio was determined by dividing the A20 particle titer by the average genomic titer.

To determine whether any mutations affected DNA packaging, the titer of DNA containing virions was determined by real-time (RT) PCRTM (Clark *et al.*, 1999; Veldwijk *et al.*, 2002) (Table 5) and confirmed by DNA dot blot hybridization. Although there was variation between preparations, the majority of the capsid mutants were able to package detectable DNA (Table 5). As expected, H358A was negative for DNA packaging, as it did not produce virus particles. It was concluded that none of the capsids in the mutant panel that made A20 positive particles were completely defective for DNA packaging. However, by comparing the A20 ELISA and PCR titers, it was noted that stocks of mutant R459A contained 40-fold more empty particles than wild type rAAV2. Thus, R459 could have a role in DNA packaging. Although less dramatic, mutants R447A, R566A, R587A, R585K, and R585K/R588K had approximately 10-fold more empty particles than rAAV2. The remainder of the virus preparations packaged DNA at levels comparable to wild type AAV2 (Table 5).

5.2.2.3 IN VITRO HEPARIN BINDING OF CAPSID MUTANTS

To assess the ability of mutant capsids to bind heparin sulfate, a modification of an assay previously described by Wu *et al.* (2002) was used. Virus preparations that had been purified by iodixanol step gradients were loaded on heparin agarose columns and the entire volume of the flow through, wash, and eluate fractions were pooled separately, denatured, and slot blotted onto nitrocellulose for immunoblot analysis with B1 antibody. A representative Western analysis for each mutant is shown in FIG. 8. As expected, wild type

AAV2 was not observed in the flow through or wash fractions and most of the virus bound to the column was recovered at the elution step. Eight other mutants, R447A, R459A, H509A, R513A, K544A, K566A, N587A, and H526A/K527A, had a heparin-agarose binding phenotype indistinguishable from wild type. The results with R513A confirmed a previous report (Wu *et al.*, 2000) in which a double mutant at positions 513 and 514 was positive for heparin binding. In marked contrast, it was observed that any capsid harboring a non-conservative mutation at position 585 or 588 was detected only in the flow through and wash. Intermediate heparin-agarose binding phenotypes in mutants R484A, R487A and K532A were also detected with approximately equal levels of signal detected in the flow through, wash, and eluate. The results with K532A were inconsistent with previous results in which a mutant containing alanine substitutions at positions 527 to 532 was found to be positive for heparin binding (Wu *et al.*, 2000). These data suggested that at least five amino acids had the potential to contribute to the electrostatic attraction between AAV and heparin sulfate. These included predominantly R585 and R588, and to a lesser but detectable extent, R484, R487, K532.

To confirm that the charge at R585 and R588 was primarily responsible for heparin interaction, two viruses were generated with conservative mutations, R585K and R585K/R588K, and tested them in the *in vitro* heparin binding assay. Both lysine and arginine residues are positively charged, however, lysine is slightly larger due to an additional methyl residue in the side group. Both of these capsids bound to heparin-agarose almost as well as wild type virus (FIG. 8). In each case, most of the virus was recovered in the eluate; however, the flow through and wash fractions also contained minor amounts of virus. This result suggested that both localized negative surface charge, and the relative position of the changes in this region of the capsid, are responsible for mediating the interaction with heparin-agarose.

Finally, as a control and to validate the heparin binding assay, the ability of wild type rAAV2, rAAV1, and rAAV5 to bind to heparin-agarose was compared. For this purpose, recombinant viruses were produced and purified by using a pseudotyping protocol developed to package AAV2 terminal repeat containing genomes into alternative serotype capsids (FIG. 9A) (Rabinowitz *et al.*, 2002; Zolotukhin *et al.*, 2002). Approximately equal amounts of input virus as determined by Western blot signal intensity were applied to a heparin-agarose column, and fractions from the column were slot blotted onto nitrocellulose for immunodetection using the B1 antibody (FIG. 9B). As expected, rAAV2 was efficiently retained by heparin-agarose under low ionic conditions but the majority of rAAV1 and all of

rAAV5 was seen in the flow through and wash. A low amount of AAV1 was detected in the eluate. These data were consistent with previous reports (Rabinowitz *et al.*, 2002).

5.2.2.4 MULTIPLE MUTATIONS IN THE AAV2 CAPSID EFFECT VIRAL TRANSDUCTION

To determine how the heparin-agarose binding phenotypes correlated to infectivity, iodixanol stocks were tested for their ability to transduce HeLa C12 cells by performing a green cell assay (GCA). Cells in a 96 well plate were co-infected with Ad5 at a constant MOI=10 pfu/cell and mutant AAV virus stocks in a 10-fold dilution series. Twenty-four hours post-infection (hpi), the number of GFP expressing cells in individual wells were counted and a GCA titer was calculated (Table 5). The detection limit of this assay was approximately 10^4 transducing units/ml. The GCA titers were then normalized to genome containing physical particles by calculating a particle to infectivity (P/I) ratio. This ratio is equivalent to the number of genomes required to transduce one cell (Table 5). To get a measure of the relative impact of a particular mutation on viral infectivity, the P/I ratio of each mutant was divided by the wild type capsid P/I ratio and the $\log_{10}$ of this value was plotted in FIG. 10. This provided a simple comparison of how many genome-containing particles of each mutant were required to achieve the same number of transduced cells as the wild type virus.

Several phenotypes emerged from this analysis. Mutants R477A, K544A, and K566A were virtually identical to wild type, and mutants R513A, N587A, R585K, and R585K/R588K were only slightly defective (approximately 1 log). These seven mutants were found previously to bind heparin sulfate to the same extent as wild type rAAV2 (FIG. 8).

Three of the mutants R459A, R484A, and K532A produced virus that was essentially non-infectious with P/I ratio between 7.2×10^4 and 3.6×10^6 compared to the wild type ratio of 25 (Table 5, FIG. 10). The P/I ratios for these mutants were minimum estimates based on the GCA assay sensitivity of 1×10^4 IU/ml. In fact, no transduction events were seen with any of these mutants.

R459A was the most severe example of three mutants (R459A, H509A, and H526A/K527A) that were essentially wild type for heparin binding but defective for transduction (FIG. 10). These mutants were presumably defective in some late stage of viral infection.

Finally, all five of the mutants that were defective or partially defective for heparin binding (R484A, R487A, K532A, R585A, and R588A) were defective for transduction.

However, the loss of infectivity did not correlate completely with the loss of heparin binding (compare FIG. 8 and FIG. 10). Two of these mutants (R484A and K532A) were only partially defective for heparin binding but severely defective (>5 logs) for transduction, suggesting that some other step in viral infection was defective in these mutants in addition to heparin binding. The remaining heparin binding mutants (R487A, R585A, and R588A) had defects in transduction that approximately correlated with their ability to bind heparin.

5.2.2.5 EVALUATION OF R585A/R588A CELL ATTACHMENT *IN VIVO*

As mentioned earlier, alanine substitutions at either position 585 or 588 were the only mutations that completely abolished binding to HS (FIG. 8), suggesting that these two arginines were primarily responsible for heparin binding. Moreover, the extent to which mutation of either or both of these residues inhibited transduction (FIG. 10, 1.5-3 logs) was approximately the same when soluble heparin sulfate is used to inhibit wild type rAAV2 infection (Handa *et al.*, 2000). Those mutants were, therefore, examined in more detail.

To see if the defect in transduction of R585 and R588 mutants could be overcome by using higher input MOI's, cells were co-infected with rAAV2 or the mutant viruses at an MOI=500 genome containing particles/cell. Twenty-four hours post-infection cells were examined by fluorescence microscopy and counted by FACS. The data from three independent experiments and representative histograms are shown in FIG. 11. As expected, the defects in transduction of the single mutants, R585A and R588A, could be overcome by higher MOI's (56% and 25% transduction for R585A, and R588A, respectively). Predictably, the level of recovery of the double mutant, R585A/R588A, was lower (10% transduction). However, it was clear that the fluorescence intensity profile for the heparin binding mutants was quite different from wild type, suggesting a significant delay in the onset of GFP expression by 24 hours. In contrast, the level of transduction of the conservative double mutant, R585K/R588K, and the heparin positive mutant, N587A, was indistinguishable from wild type.

As a more direct assay for cell attachment, Hela C12 cells were transfected and the location of viral DNA tracked. Cells were infected with rAAV2 at an MOI=100 or R585A/R588A at an MOI=1000 genome containing particles as determined by RT-PCR™. At 1, 4, and 20 hpi, the infection media was removed and saved, and the cells were washed extensively to remove any residual unbound virus. The cells were then harvested and low molecular weight DNA was extracted from both the infection media (unbound) and the cell

pellet (bound or internalized) by the Hirt procedure and transferred to nitrocellulose for Southern hybridization with an [α - ^{32} P]-dATP labeled GFP probe (FIG. 12A and FIG. 12B).

At all time points rAAV2 DNA was detectable both bound/internalized and in the infection media. In contrast, cells infected with 10-fold more genomic copies of R585A/R588A showed the vast majority of the signal only in the unbound fraction (FIG. 12A). Phosphor imager analysis determined that at each time point approximately one third of the total rAAV2 DNA was attached or internalized compared to only 1% of R585A/R588A (FIG. 12B). As these infections were performed at 37°C, the process of internalization should not have been prevented. This result demonstrated that the block in infection for mutant R585A/R588A occurred at the cell attachment stage or internalization stage, and correlated to heparin sulfate binding *in vitro*.

5.2.2.6 LOOP SWAPPING CONFERS HEPARIN BINDING TO AAV5

Although the primary amino acid sequences are moderately divergent, the architectural position of β -sheets and loops is predicted to be very similar among AAV serotypes (Rabinowitz and Samulski, 2000). It was hypothesized that if R585 and R588 were the critical residues involved in HSPG binding, then it should be possible to substitute that region of AAV2 into AAV5 to create a hybrid virus capable of interacting with heparin-agarose. To achieve this, a recombinant virus, designated rAAV5-HS, was generated by replacing a short loop containing residues 585 through 590 from AAV2 into a region predicted to be structurally equivalent in AAV5 (FIG. 13A). Loop substitution rather than point mutagenesis was done to account for the possibility of additional Van der Waals interactions or hydrophobic contributions from nearby amino acids.

Production and purification of rAAV5-HS was unaffected by the six amino acid substitution (FIG. 13B; Table 5). When rAAV5-HS was tested in the *in vitro* heparin-agarose binding assay, it was indistinguishable from wild type rAAV2 (FIG. 7C). These data suggested that this region of AAV5 was surface accessible, and that heparin-agarose binding could be artificially conferred by the six amino acids containing R585 and R588.

To compare the infectivity of rAAV5 and rAAV5-HS, packaged viruses were generated that contained a recombinant AAV5 genome in which the GFP reporter gene was flanked by AAV5 terminal repeats. The infectivity of these viruses was compared to rAAV2 in a GCA assay and particle-to-infectivity ratios were calculated as before (FIG. 13D). rAAV5 was able to transduce Hela C12 cells at a low efficiency, approximately 2.5 logs

lower than AAV2. However, no transduction was seen with AAV5-HS ($<1 \times 10^4$ IU/ml) (Table 5; FIG. 13D). Given the minimum sensitivity of the GCA assay this meant that the P/I ratio of AAV5-HS was at least 3.5 logs higher than rAAV2 and at least 1 log higher than wild type rAAV5. It was concluded that, although substitution of these five heterologous amino acids into the AAV5 capsid restored heparin binding to the level of AAV2 capsids, it was not sufficient to produce AAV2 levels of infectivity in a cell line normally permissive for AAV2.

5.2.3 DISCUSSION

This example describes the identification of amino acids in the capsid of AAV2 that mediate binding to heparin sulfate proteoglycans. Several lines of evidence suggest that HSPG serves as the primary receptor for AAV2. Inhibition of AAV2 infection can be demonstrated by competition with soluble analogs, GAG desulfation by sodium chlorate treatment, antibody competition, enzymatic removal of heparin, and use of mutant cell lines that express varying levels of HSPG (Handa *et al.*, 2000; Qiu *et al.*, 2000; Summerford and Samulski, 1998; Wu *et al.*, 2000). Binding to heparin sulfate is usually the result of electrostatic charge interactions between basic amino acids (R, K, or H) and negatively charged sulfate residues (Hileman *et al.*, 1998; Mulloy and Linhardt, 2001). During the course of previous mutagenesis studies, many of the basic amino acids in the AAV2 capsid that could potentially contribute to heparin sulfate binding were eliminated (Wu *et al.*, 2000). In this example, the remaining basic residues were examined by looking at their conservation in AAV serotypes 1-5. Those that were present in all five serotypes were not likely to contribute significantly to heparin binding. Those that were conserved in the heparin binding serotypes, AAV1-3, but not in the remaining serotypes were targeted for mutagenesis. Finally, by taking advantage of the fact that R585 and R588 had been previously identified as potential heparin binding amino acids (Wu *et al.*, 2000) and that these amino acids were located in a cluster of basic residues at the three fold axis of symmetry (Xie *et al.*, 2002), all of the basic amino acids in this cluster were also targeted for mutagenesis. This approach yielded a total of 15 amino acids that could have been involved in heparin binding and alanine mutations were characterized at all of these positions. This approach, of course, does not necessarily identify *all* possible heparin binding amino acids. For example, R484, which is basic in all five serotypes was tested because of its proximity to R585 and R588 and subsequently proved to be involved in heparin binding.

5.2.3.1 HEPARIN BINDING AND INFECTIVITY

These studies indicated that capsids with mutations at residue 484, 487, 532, 585 or 588, were partially or completely defective for heparin-agarose binding. The most severe defect was found with mutations in R585 and R588. No binding to heparin sulfate columns could be detected with either mutant (FIG. 8), and both mutations reduced the particle-to-infectivity ratio by two to three logs (Table 5). Mutants that contained substitutions at both positions had even lower infectivity.

The phenotypes of R487A, R585A, and R588A, were probably due largely to defective heparin binding. For example, the double mutant R585A/R588A was approximately 500 fold more defective in cell binding and internalization than wild type (FIG. 12B) when corrected for the MOI, and approximately 2000 fold less infectious (Table 5), as judged by the change in particle-to-infectivity ratio. Another indication that heparin binding was primarily responsible for the defects in R585 and R588 was the fact that conservative mutations at these two positions (R585K and R585K/R588K) produced virus particles with properties similar to wild type (FIG. 8; FIG. 10; FIG. 11; Table 5). Results from the conservative lysine substitutions at R585 and R588 are reasonably consistent with electrostatic attraction being the primary mediator for AAV-heparin interaction. R585K, the least defective heparin binding mutant (FIG. 8), had transduction levels nearly equal to rAAV2 (FIG. 10), and R585K/R588K was only slightly more defective for heparin binding (FIG. 8) and transduction (FIG. 10), and within one log of wild type. Furthermore, when cells were infected at a high MOI, robust transduction was observed for both mutants (FIG. 11). Finally, substitution of a six amino acid sequence containing R585 and R588 imparted heparin binding to AAV5 that was comparable to that seen with AAV2 (FIG. 13). Although similar studies were not performed with the R487 position, it was clear that mutation of R487 produced virus with a more modest defect in heparin binding (FIG. 8) and in infectivity (FIG. 10).

In addition to R487A, R585, and R588, two other mutants were found that were defective for heparin binding, R484A and K532A. R484A and K532A, like R487A, had a more modest effect on binding to heparin sulfate, but unlike the other heparin binding mutants, these two mutations had a dramatic effect on transduction efficiency. Both R484A and R532A were more than 5 logs less infectious than wild type capsids (Table 5; FIG. 10). This severe defect is presumably due to a different block in the infection process that is unrelated to heparin binding, but as yet it has not been identified. The result from K532A is consistent with earlier studies that identified a mutant (mut 37) that contained six amino acid

substitutions that included K532A (Wu *et al.*, 2000). Mut 37 had a phenotype identical to K532A in that it produced full virus particles that were non-infectious and more recently has been shown to have a modest defect (approximately 5 fold) in heparin binding and internalization. This potentially maps this defect to a single amino acid.

5.2.3.2 COMPUTER MODELING

Using the recently published atomic structure of AAV2 (PDB ID code: 1LP3) (Xie *et al.*, 2002), the positions of the heparin binding mutations were examined. Symmetry transformation operations from the original PDB file were applied to generate a VP3 trimer arrangement in the context of an icosahedron. When viewed in ribbon format looking directly down a three-fold axis, residues R484, R487, R532, R585 and R588, represented as balls-and-sticks, are located in a linear formation lining one side of each three-fold related spike. When viewed across the top surface of the trimer, residues R585 and R588, which are contributed by one of the peptides in the trimer, are positioned above a linear arrangement of R484, R487 and K532, which are contributed by a second peptide in the trimer. Thus, it appears that a heparin binding motif is formed from some combination of these five amino acids using amino acids from two different polypeptides. An electrostatic potential surface map of a VP3 trimer was also generated, in which areas of positive and negative charge are represented. When viewed perpendicular to the three fold-axis, the five amino acids mapped by this example appear to contribute collectively to a basic patch on one side of each three-fold related spike. The charge, clustering, and surface presentation of these residues are all consistent with a model of electrostatic attraction. Two other basic residues, H526 and K527, contribute to the basic cluster at the three fold spike but these residues do not appear to be involved in heparin binding (FIG. 8).

The five mutations that affected heparin binding were located in the large loop IV region, which among AAV serotypes has low overall sequence conservation and includes all of the previously identified insertion and substitution mutations that affect heparin binding. Interestingly, with the exception of N587, the stretch of amino acids encompassing 585 to 590 is unique to AAV2 and is not present in AAV3, which is the other AAV serotype that has been shown to bind efficiently to heparin sulfate. Mutation of N587 had no effect on heparin-agarose binding and only minor effects on transduction. Conceivably, residues R484, R487 and K532 could be the dominant residues involved in heparin sulfate binding for AAV3.

The apparent dissociation constant (K_d) of AAV2 and heparin sulfate was determined by competition analysis to be 2×10^9 M (Qiu *et al.*, 2000). Although this is higher than some heparin-protein interactions, it is sufficiently strong to suggest cooperative binding by one HS glycosaminoglycan chain to multiple attachment points. This example does not address whether heparin sulfate could form a bridge between basic residues in one of the threefold spikes to those in another. However, as the average chain length of heparin glycosaminoglycans varies between 50-200 disaccharide repeats that adopt a helical conformation 40-160 nm in length, it is conceivable that a heparin sulfate chain could wrap around the exterior of the capsid through cooperative binding of multiple spikes at the threefold axis of symmetry. Although a rigorous computational docking analysis was not undertaken, a heparin molecule (PDB ID code 1NTP) was manually superimposed in several orientations that placed multiple reactive sulfate and amine groups within accepted electrostatic attraction distances on pairs of residues spanning the spikes.

5.2.3.3 MUTANTS THAT BIND HEPARIN BUT ARE STILL DEFECTIVE

Several new mutants were found that bound heparin sulfate as well as wild type but still produced defective particles. H538A was defective for particle assembly. There are a number of reported examples of mutations that disrupt AAV2 particle formation, several of which are located in the conserved β -strand regions (Rabinowitz *et al.*, 1999; Shi *et al.*, 2001; Wu *et al.*, 2000). Since H358 is neither surface accessible nor in a conserved β -strand, it is possible that it acts internally to stabilize the monomer subunit structure.

Mutants R459A, H509A, and H526A/K527 bound heparin-agarose efficiently but had particle-to-infectivity ratios that were two to more than three logs higher than wild type. Like K532A and R484A, these mutants are presumably defective in some stage of the infectious entry pathway between secondary receptor binding and uncoating. Ongoing studies in the lab are examining the block in infectivity for these mutants.

5.2.3.4 DNA PACKAGING

The process of DNA packaging is thought to occur by an active process requiring NTP consumption coupled to the helicase activity of the small Rep proteins (King *et al.*, 2001). Although none of the mutations that assembled an A20 positive particle were completely deficient for DNA packaging, mutant R459A produced a 40-fold excess of empty capsid particles compared to rAAV2. Other studies have reported that short insertions at

positions 323, 339, 466, 520, 540, 595, 597 that did not interfere with capsid formation still reduced DNA packaging to levels detectable only by PCR™ amplification (Shi *et al.*, 2001). In addition, a point mutant R432A prevents DNA packaging (Wu *et al.*, 2000). Although the relationship between these mutations and their mechanism of action is unclear, it is possible that they disrupt protein-capsid or DNA-capsid interactions.

5.2.3.5 SUMMARY OF EXEMPLARY PRODUCTION SYSTEM

An exemplary rAAV production system has been described to produce modified rAAV vectors that comprise one or more altered capsid proteins. FIG. 14 shows the results of an immunoslotblot of total capsid protein following standard purification procedures of a representative expression system of the invention. FIG. 15 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and the system plasmid combinations. FIG. 16 shows the *in vivo* transduction ability of recombinant AAV vectors produced using various system components. FIG. 17 shows an Immunoblot and dot blot autoradiograph of virions produced from pTR-UF5; pIM45-VP1,2; pIM45-VP1,3; and pIM45-VP2,3 plasmids following standard purification protocols. FIG. 18 shows the *in vivo* transduction ability of recombinant AAV vectors containing only two capsid proteins, while FIG. 19 depicts an immunoblot of protein fractions collected from iodixanol purified passed over a heparin-agarose column. Using an anti-VP1,2,3 monoclonal antibody. FIG. 20 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and rAAV R585A, R588A, while FIG. 21 summarizes an exemplary system that demonstrates the *in vivo* transduction ability of pTR-UF5 and R585A, R588A. FIG. 22 shows a slot blot autoradiograph of an *in vivo* DNA tracking time course experiment of pTR-UF5, rAAV R585A, R588A, while FIG. 23 shows a schematic diagram of the pIM45 vector showing the rep and cap sequences.

5.3 EXAMPLE 3 – THE ADENO-ASSOCIATED VIRUS 2 VP2 CAPSID IS NON-ESSENTIAL AND CAN TOLERATE LARGE PEPTIDE INSERTIONS AT ITS N-TERMINUS

Interest in the composition, assembly, and atomic structure of the AAV particle stems in part from its promise as a recombinant gene delivery vehicle *in vivo*. However, further clinical development of AAV for gene therapy will require the ability to target specific tissue types. A better understanding of the particle's surface architecture has been obtained through systematic alanine-scanning (Wu *et al.*, 2000) and insertional mutagenesis (Girod *et al.*, 1999; Rabinowitz *et al.*, 1999; Shi *et al.*, 2001) of the AAV *cap* ORF and

determination of the atomic structure of AAV (Kronenberg *et al.*, 2001; Xie *et al.*, 2002). These studies have identified several regions on the particle surface that tolerate the insertion of foreign sequences. Thus far, small changes in size, sequence, and/or position of the insertion have resulted in unpredictable changes in the mutant particle phenotype. Nevertheless, direct insertion of targeting sequences into the *cap* ORF has resulted in the successful production of AAV vectors with both expanded and retargeted tropisms (Buning *et al.*, 2003). In particular, the insertion of targeting sequences in the VP1/2 and VP3 capsid overlap regions of the *cap* ORF (immediately following residue 138 or 587) have produced AAV with alternative cellular receptor usage. Insertions after residue 138 (N-terminus of VP2) expand the tropism of AAV (Loiler *et al.*, 2003; Shi *et al.*, 2001; Wu *et al.*, 2000), as they do not disturb the capsid residues involved in binding cellular heparan sulfate proteoglycan (Kern *et al.*, 2003; Opie *et al.*, 2003). Ligands inserted after residue 587 (Girod *et al.*, 1999; Grifman *et al.*, 2001; Muller *et al.*, 2003; Nicklin *et al.*, 2001; Perabo *et al.*, 2003; Ponnazhagan *et al.*, 2002; Rabinowitz *et al.*, 1999; Ried *et al.*, 2002; Shi *et al.*, 2001; Shi and Bartlett, 2003; Wu *et al.*, 2000) reside at the particle's threefold axis between critical residues involved in cell binding via heparan sulfate proteoglycan (Kern *et al.*, 2003; Opie *et al.*, 2003; Xie *et al.*, 2002), the primary viral receptor. Thus, these insertions can simultaneously restrict viral entry and redirect it to an alternative receptor. Still, these inserted sequences have been restricted in size (~30 amino acids) consisting of linear receptor binding epitopes. One limitation to manipulating the *cap* ORF in the direct insertion approach is that modification of only one capsid across its entire sequence, leaving the remaining two capsids unaltered, is not possible. Only one region of the *cap* ORF allows for modification of a single capsid (VP1, residues 1-137) and this region contains a phospholipase A motif that is critical for efficient viral infection (Girod *et al.*, 1999). A single report (Yang *et al.*, 1998) has shown that a significantly larger single chain antibody coding sequence can be incorporated into recombinant particles if it is fused to the N-terminus of VP2 and co-expressed with wild type VP1, VP2, and VP3 capsids. These particles were capable of retargeting the vector to the CD34 molecule but recombinant titers were extremely low.

In this example, using missense mutation of *cap* start codons, plasmids were generated that expressed only one or two of the capsid proteins, and their ability to produce AAV particles was tested. AAV-like particles are produced as long as VP3 is present.

Characterization of the physical titers of these AAV-like particles that lacked specific capsid proteins demonstrated that the VP2 protein is apparently redundant and is not essential for viral infectivity. Importantly, using these constructs, a method of producing AAV-like particles with large peptide insertions in VP1 and VP2 or VP2 exclusively was described, by expressing the modified protein separately, and providing the remaining wild type capsids *in trans*. Finally, AAV-like particles could be produced with altered capsid composition if VP2 is significantly over-expressed.

5.3.1 MATERIALS AND METHODS

5.3.1.1 PLASMIDS

Plasmid, pIM45, contains the *rep* and *cap* coding sequences of AAV with their expression controlled by their native promoters (McCarty *et al.*, 1991). It was used as a parent template for construction of all mutant plasmids. Plasmid pXX6 (Xiao *et al.*, 1998) supplies the adenovirus helper gene products *in trans* to allow rAAV production in an adenovirus free environment and was supplied by Jude Samulski. Plasmid pTR-UF5 (Zolotukhin *et al.*, 1996) supplies the rAAV DNA to be packaged. It contains a cytomegalovirus promoter driving expression of a GFP reporter gene flanked by the AAV terminal repeats. Plasmid pTRdsRed is identical to pTR-UF5 except that the GFP coding sequence is substituted with the red fluorescent protein (RFP) coding sequence.

5.3.1.2 CONSTRUCTION OF MUTANT PLASMIDS

Site directed mutagenesis (Stratagene) was performed on plasmid pIM45 as per the manufacturer's instructions. For each mutant plasmid, two complementary PCR™ primers containing a missense mutation in the individual capsid protein start codons were used to introduce changes in the *cap* ORF of pIM45. The oligonucleotides used for mutagenesis are listed in Table 6. These plasmids were screened for restriction sites inserted by silent mutations, and the mutations were confirmed by DNA sequencing.

TABLE 6
SEQUENCES OF OLIGONUCLEOTIDES USED FOR MUTAGENESIS

Name	Sequences (5' to 3')
VP1-M1L ^a	gatttaa ~~at~~ caggtCTGgctgccgatgggtatcttccagattggctcg (SEQ ID NO:1)
VP2-T138A	ggaaccgggtaagGCGgctccgggaaaaaagaggccggt (SEQ ID NO:2)

Name	Sequences (5' to 3')
VP2-T138M	ggaaccgggtaagATGgctccgggaaaaaagaggccggt (SEQ ID NO:3)
VP3-M203L	cccctctggcctaggaactaatacgCTGgctacaggcagtggcgc (SEQ ID NO:4)
VP3a-M211L	gctaccggtagtggcgcaccaCTGgcagacaataacgagggcgcc (SEQ ID NO:5)
VP3b-M235L	tggcattgcgattccacatggCTGggcgacagagtcaccacc (SEQ ID NO:6)
pIM45-E/M138	aggaacctgttaagacgCGGCCGACGCGTgctccgggaaaaaagag (SEQ ID NO:7)
VP2A-E/M138	aggaacctgttaagATGCGGCCGACGCGTgctccgggaaaaaagag (SEQ ID NO:8)
FKN insert ^b	cgCGGCCGtctggttcaggtagcggttctggtcagcacctcggcacgaaatgc (+) (SEQ ID NO:9)
	cgACGCGTaccgctgccagaacctgagccgctaccatttctagtcagggcagcggt (-) (SEQ ID NO:10)
LEP insert	cgCGGCCGgtgcccacccaaaaagtccaagat (+) (SEQ ID NO:11)
	cgACGCGTgcacccagggctgaggtccagctg (-) (SEQ ID NO:12)
GFP insert	cgCGGCCGatgagcaagggcgagggaactg (+) (SEQ ID NO:13)
	cgACGCGTctgtacagctcgccatgcc (-) (SEQ ID NO:14)

^a top group: + complementary oligonucleotide

^b bottom group: (+) sense; (-) antisense

5.3.1.3 CONSTRUCTION OF AAV CAPSID MUTANT PLASMIDS FOR DIRECTIONAL CLONING OF INSERTIONS AT AMINO ACID POSITION 138

The same site-directed mutagenesis strategy was used to insert an *EagI*/*MluI* cloning site immediately after amino acid position 138 in pIM45. The same oligonucleotide pair with an additional T138M mutation was used to introduce these sites into pVP1,2A and pVP2A. The resulting plasmids were called pIM45-E/M138, pVP1,2A-E/M138, and pVP2A-E/M138. The cDNA for the rat fractalkine chemokine domain (FKN, CX3CL1, accession: NM134455), the human hormone leptin (LEP, accession: BC060830), and the green fluorescent protein (GFP, accession: U50963) flanked by *EagI* and *MluI* restriction sites were generated using PCR[™] (Table 6). The PCR[™] products were cloned into pIM45-E/M138, pVP1,2A-E/M138, and pVP2A-E/M138.

5.3.1.4 CELL CULTURE

Human embryonic kidney 293 and cervical carcinoma HeLa C12 cells (Clark *et al.*, 1996) were grown in Dulbecco Modified Eagle Medium (Invitrogen) supplemented with 100U/ml penicillin, 100U/ml streptomycin, 10% bovine calf serum, sodium pyruvate, and 2 μ M glutamine. Cells were incubated at 37°C in a 5% CO₂ atmosphere.

5.3.1.5 PRODUCTION OF AAV PARTICLES

To produce AAV virions with wild type capsid proteins, low passage 293 cells were transfected at ~80% confluence using a modification of the triple transfection protocol (Li *et al.*, 1997; Xiao *et al.*, 1998; Zolotukhin *et al.*, 1996). All plasmids were transfected in equivalent molar ratios using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's suggestions. One or two pIM45-based plasmids carrying the appropriate capsid protein mutation(s) or ligand insertions, pXX6, and either pTRUF5 or pTR-dsRed (total DNA=70-90 μ g) were transfected into three 15 cm² dishes and 24 hrs later transfection efficiency was determined using fluorescent microscopy. Efficiencies were consistently above 75% with this method. The three dishes were then pooled and vector purification was carried out as previously described using an iodixanol step gradient alone or in combination with heparin column chromatography (Hermens *et al.*, 1999; Zolotukhin *et al.*, 1999; Zolotukhin *et al.*, 2002).

5.3.1.6 VIRUS TITER DETERMINATION

To determine the concentration of intact AAV particles, the A20 ELISA (American Research Bioproducts) was used. The A20 antibody detects intact, fully assembled particles, both full and empty (Grimm *et al.*, 1999; Grimm *et al.*, 1998). Iodixanol purified stocks were serially diluted and processed by the manufacturer's recommended protocol. Only readings within the linear range of the assay were averaged.

To determine the concentration of DNA containing particles, real-time PCR[™] was performed (Clark *et al.*, 1999; Veldwijk *et al.*, 2002) using a Perkin Elmer-Applied Biosystems (Foster City, CA) Prism 7700 sequence detector system. Equal volumes of virus stocks were treated with 600 U/ml benzonase in 50 mM Tris-CL (pH 7.5), 10 mM MgCl₂, and 10 mM CaCl₂ at 37°C for 30 min. The reactions were adjusted to 10 mM EDTA and 5% SDS and incubated with 280 U/ml proteinase K at 37°C for 30 min. The reactions were then extracted with phenol/chloroform/ isoamyl-alcohol (25:24:1) and the packaged DNA was precipitated overnight with ethanol and glycogen carrier. The precipitated DNA pellets were dissolved in 100 μ l of water and 5 μ l was used for real-time PCR[™] analysis in a reaction mixture that included 900 nM each of GFP forward and reverse primers, 250 nM Taqman probe, and 1X Taqman universal PCR[™] master mix in a total volume of 50 μ l. The cycling parameters were 1 cycle each of 50°C, 5 min, and

95°C, 10 min, followed by 40 cycles of 95°C, 15 sec and 60°C, 1 min. Only values within the linear portion of a standard curve having a coefficient of linearity greater than 0.98 were accepted. The average real-time PCR[™] titer was calculated from virus preparations assayed three times.

For AAV particles with GFP inserted in VP1 and VP2 or VP2 exclusively, the RFP gene from pTR-dsRed was packaged and particle titers were determined by dot blot as described previously (Zolotukhin *et al.*, 1999). Equal volume aliquots of the vector preparations were incubated with DNaseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. The DNA was then transferred to nitrocellulose and probed with radiolabelled RFP probe.

To determine the infectious titer of the wt and mutant virus stocks, a fluorescent cell assay (FCA) was performed essentially as previously described (Zolotukhin *et al.*, 1999). Briefly, HeLa C12 cells were seeded in a 96-well plate so that they were approximately 75% confluent at infection. Cells were infected with 10-fold serial dilutions of the vector preparations and Ad5 at a multiplicity of infection (MOI) of 10. Cells were incubated at 37°C in a 5% CO₂ atmosphere for 24 hours and examined by fluorescence microscopy. The average FCA titer was calculated by averaging the number of green fluorescent cells (or red fluorescent cells in the case of virus that contained a GFP insert in the particle) from preparations assayed three times. Particle to infectivity ratios were calculated by dividing the average DNA titer by the average FCA titer.

5.3.1.7 CONFOCAL MICROSCOPY OF AAV-LIKE PARTICLES WITH GFP INSERTED IN VP1 AND VP2

HeLa cells were seeded in 8 chamber tissue culture slides (Falcon) 24 hours prior to infection with VP1,2A-GFP particles at an MOI of 10,000 in the absence and presence of Ad 5 (MOI=20). Tissue cultures were fixed in 4% ice-cold para-formaldehyde solution for 4 hr. To reduce non-specific labeling, the slides were incubated in 1% bovine serum albumin (BSA) in 0.01 M Phosphate buffered saline (PBS, pH 7.2-7.4) for 1 hr at room temperature (RT). The primary rabbit anti-Early Endosomal Antigen 1 (EER1) antibody (Novus Biologicals, Inc. Littleton, CO.), which was diluted at 1:1000 with 0.1% BSA and 0.3 % triton in PBS, was incubated for 24 hr at 4°C. The secondary antibody, Cy⁵-conjugated donkey anti-rabbit IgG at a 1:100 dilution in PBS (Jackson ImmunoResearch Laboratories, West Grove, PA) was applied for 1 hr at RT. Between each incubation step,

slides were rinsed in PBS for 30 min at RT. For propidium iodide (PI) staining, the slides were briefly equilibrated in 2xSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) and incubated in 100 μ g/ml DNase-free RNase in 2XSSC for 20 min at 37°C. The slides were then coverslipped using Vectashield mounting medium with PI (Vector Laboratories, Inc. Burlingame, CA). Sections were examined with a confocal laser scanning microscope (Bio-Rad Olympus) illuminated by three lasers (argon, “green” helium–neon, and “red” helium–neon), which supply excitation lines at 458, 488, 514, 543, and 633 nm. This allowed simultaneous confocal imaging of the three fluorophores (*i.e.*, GFP, PI and Cy5). Cells on each slide were examined first for GFP staining. The focal plane was adjusted so that the number of detectable cell bodies was maximized and the green GFP image was then stored in memory. The procedure was repeated for the red PI image and the blue Cy5 image. Finally, a superimposition of the three colored images was made and stored. All manipulations of contrast and illumination on color images were made using Adobe PhotoShop® 6.0 software on a PC.

5.3.2 RESULTS

5.3.2.1 DIRECT INSERTION OF LARGE PEPTIDES AFTER RESIDUE 138 OF THE AAV CAPSID ORF DOES NOT YIELD PARTICLES

Residue 138 was chosen because ligands inserted at this position are present on the surface of the particle and result in alternative receptor recognition by AAV vectors (Loiler *et al.*, 2003; Shi *et al.*, 2001; Wu *et al.*, 2000). Furthermore, this position does not directly interrupt the phospholipase A2 motif of VP1 (Girod *et al.*, 1999) or interfere with the structurally critical VP3 β -barrel arrangement (Xie *et al.*, 2002). To test the direct insertion of larger peptides into *cap*, the directional cloning sites *EagI* and *MluI* were inserted immediately after residue 138 of the *cap* ORF in the plasmid pIM45, which contains the wild type *rep* and *cap* sequences. The choice of these restriction enzymes meant that ligands inserted into the resulting plasmid (pIM45-E/M138) were flanked by arg and pro on the N-terminal side and arg and thr on the C-terminal side. These additional four amino acids had little effect on capsid expression, particle formation or titers (FIG.24A and FIG. 24B, Table 7). The 8 kDa FKN (76 residues) and the 18 kDa LEP (146 residues) coding sequences were chosen because they are approximately half (FKN) or the same (LEP) size as the VP1 N-terminal extension of VP2 (137 residues). These sequences were inserted into pIM45-E/M138 and the resulting plasmids, pIM45-FKN138

and pIM45-LEP138, were transfected into 293 cells in the presence of Ad5 (MOI=10). Western blot analysis of equivalent volumes of 293 whole cell lysates with B1 antibody, which recognizes a linear epitope in the C-terminal region of all three capsid proteins (Wobus *et al.*, 2000), showed a severe loss of the most abundant capsid protein, VP3 (FIG. 24A). In addition, the expression level of the modified VP2 also appears to decrease with the larger LEP insertion. Both VP1 and VP2 had the expected increased molecular weight due to the insertion of FKN and LEP.

As expected, this aberrant capsid protein expression did not result in the formation of AAV particles. Following transfection of pIM45-E/M138, pIM45-FKN138, or pIM45-LEP138 with pXX6 and pTR-UF5, particles were purified by iodixanol density gradient centrifugation. In contrast to the parental plasmid pIM45-E/M138, essentially no particles were recovered from cells transfected with pIM45-FKN138 or pIM45-LEP138 (FIG. 24B, Table 7). The parental plasmid pIM45-E/M138, which had a 4 amino acid insertion in VP1 and VP2 produced virus with approximately the same yield of particles and particle to infectivity ratio as pIM45, which contained wild type capsid proteins.

TABLE 7
PROPERTIES OF AAV AND AAV-LIKE PARTICLES

Virus	Particle titer ^a		Infectious titer (IU/ml) ^b	Particle to infectivity ratio ^c	Empty/full ratio ^d
	A20/ml	Genomes/ml			
<i>VP3 N-terminus</i>					
WT	7.2×10^{12}	3.6×10^{11}	1.8×10^{10}	20	20
M203L	<i>No Virus</i>				
M211L	<i>No Virus</i>				
M235L	2.9×10^{12}	2.2×10^{11}	9.0×10^9	24	13
<i>(-) capsid proteins</i>					
VP1,2	<i>No Virus</i>				
VP1,2A	<i>No Virus</i>				
VP1,3	6.2×10^{12}	1.0×10^{11}	4.6×10^9	22	62
VP2,3	6.7×10^{12}	1.4×10^{11}	4.5×10^9	31111	48
VP2A,3	2.0×10^{12}	4.0×10^{10}	9.0×10^4	444444	50
VP1	<i>No Virus</i>				
VP2	<i>No Virus</i>				
VP2A	<i>No Virus</i>				

Virus	Particle titer ^a		Infectious titer (IU/ml) ^b	Particle to infectivity ratio ^c	Empty/full ratio ^d
	A20/ml	Genomes/ml			
VP3	5.0×10^{12}	1.3×10^{11}	5.0×10^4	2600000	38
<i>Complementation</i>					
VP0 + WT	5.2×10^{12}	3.6×10^{11}	3.5×10^9	103	14
VP1 + VP2,3	4.6×10^{12}	3.4×10^{11}	1.6×10^{10}	21	14
VP2 + VP1,3	8.8×10^{12}	5.8×10^{11}	1.6×10^{10}	36	15
VP2A +VP1,3	5.8×10^{12}	3.4×10^{10}	1.8×10^8	189	170
VP3 + VP1,2	4.6×10^{12}	4.6×10^{11}	1.6×10^{10}	29	10
<i>pIM45-E/M138 inserts</i>					
E/M138	1.8×10^{12}	1.7×10^{11}	2.7×10^9	63	11
FKN138	<i>No Virus</i>				
LEP138	<i>No Virus</i>				
<i>VP1/2 peptide inserts</i>					
VP1,2A-FKN + VP3	3.9×10^{12}	6.0×10^{10}	2.8×10^5	214286	65
VP2A-FKN + VP1,3	6.8×10^{12}	1.2×10^{11}	1.4×10^9	86	57
VP1,2A-LEP + VP3	3.1×10^{12}	4.4×10^{10}	3.4×10^5	129411	70
VP2A-LEP + VP1,3	5.9×10^{12}	1.2×10^{11}	1.8×10^9	66	49
VP1,2A-GFP + VP3	2.0×10^{12}	4.0×10^9	$<1 \times 10^4$	>400000	500
VP2A-GFP + VP1,3	4.3×10^{12}	1.9×10^{10}	7.0×10^5	27143	226

^a A20 particle titers were determined as described in Materials and Methods using the A20 ELISA assay. Genomic titers were determined by RT-PCRTM.

^b Infectious titers were determined by fluorescent cell assay as described.

^c Particle to infectivity ratio was calculated by dividing the average genomic titer as determined by RT-PCRTM by the average green cell assay titer.

^d Empty to full ratio was determined by dividing the A20 particle titer by the average genomic titer.

5.3.2.3 CONSTRUCTION OF MUTANTS THAT LACK EXPRESSION OF SPECIFIC CAPSID PROTEINS

The loss of VP3 following insertion of large ligands after residue 138 suggested that VP3 would have to be provided *in trans* to complement the ligand-extended VP1 and VP2. For this purpose, a complementary capsid protein expression system was generated that would allow for a single capsid protein to be modified in a region of sequence overlap

(e.g., genetic modifications of VP2 exclusively at residue 138). To generate the necessary plasmids that expressed either one or two capsid proteins, missense mutations in the AAV *cap* ORF translational start codons were employed as reported previously by others (Muralidhar *et al.*, 1994; Ruffing *et al.*, 1992).

5.3.2.3.1 MUTANTS EXPRESSING TWO CAPSID PROTEINS

With pIM45 as a template, the VP1 start codon, M1L, was mutated generating the construct pVP2,3, which should only make VP2 and VP3 (FIG. 25A, Table 8). Similarly, the VP2 start codon, T138A, was mutated, generating the construct pVP1,3 that would make only VP1 and VP3. Finally, the VP3 start codon, M203L, was mutated in an initial attempt to generate the construct, pVP1,2 (FIG. 25A). Western blotting analysis of capsid protein expression in 293 cell lysates demonstrated that, while the expression of VP1 and VP2 were eliminated by single point mutations (Fig. 25A), pM203L expressed a VP3-like species that migrated slightly faster than VP3 (VP3a) (see FIG. 25A, lane pM203L). This had been seen previously by Ruffing *et al.* (1992), who had used a similar strategy to eliminate VP3 expression. To evaluate the role of alternative downstream translational start codons in the production of VP3a, further point mutations in met residues downstream of the native VP3 start codon were generated in the pM203L background. Examination of the VP3 coding region revealed nine additional met residues are present (M211, M235, M371, M402, M434, M523, M558, M604, and M634). Of these, only positions M211, M235, M523, M558, and M604 were in a favorable Kozak context for translational initiation. As VP3a is only slightly smaller than VP3, the role of M211 and M235 in the production of VP3-like species was initially examined. M211L was mutated alone, and with M235L on an M203L background (FIG. 25B), generating the constructs pM203,211L, and pM203,211,235L. Western blot analysis of capsid protein expression in whole cell lysates revealed that all three met residues had to be mutagenized to eliminate VP3 expression (FIG. 25B). The robust expression of VP3a was again seen with pM203L (FIG. 25B). Additionally, transfection of pM203,211L resulted in weaker expression of a second yet smaller VP3-like species, VP3b (FIG. 25B, lane pM203,211L), while expression of all VP3-like species was eliminated in the triple mutant M203,211,235L, finally generating the plasmid, pVP1,2 (pM203,211,235L), which makes only VP1 and

VP2 (FIG. 25B, lane pVP1,2). Weak doublets present at the VP3 position in the pVP1,2 lane are due to cellular proteins that cross react with the B1 antibody (data not shown).

An alternative approach to eliminating VP3 expression has been reported (Muralidhar *et al.*, 1994; Ruffing *et al.*, 1992) in which mutation of the VP2 start codon to the stronger ATG (T138M) results in loss of VP3 expression. As this approach minimizes the number of mutations in VP1 and VP2, while maximizing the expression of VP2, the VP2 start codon (T138M) was mutated on a pIM45 template, generating the construct pVP1,2A (FIG. 25C). Western blot analysis of capsid protein expression in lysates from cells transfected with pVP1,2A confirmed that this approach produced normal levels of VP1, significant over-expression of VP2, and loss of VP3 expression (FIG. 25C).

5.3.2.3.2 MUTANTS EXPRESSING A SINGLE CAPSID PROTEIN

Generation of capsid mutants that express a single capsid protein was accomplished by sequential mutation of start codons in the mutants that express two capsid proteins (FIG. 26). The construct that expressed only VP1 (pVP1) had the VP2 start codon mutated, T138A, and the M203, 211,235L mutations that were required to eliminate VP3-like species (Table 8). The construct pVP2 had the VP1 start codon mutation, M1L, and the M203,211,235L mutations, while construct pVP2A (to over-express VP2 alone) had the VP1 start codon mutation, M1L, and the VP2 start codon mutation, T138M. Finally, the construct pVP3 had the VP1 start codon mutation, M1L, and the VP2 start codon mutation, T138A. Western blot analysis of capsid protein expression in 293 cells transfected with these plasmids showed that indeed these constructs expressed only a single capsid protein as expected (FIG. 26). Finally, the construct pVP2A significantly increased expression of VP2 in the absence of VP1 or VP3 (FIG. 26).

TABLE 8

**PLASMID COMBINATIONS FOR PRODUCTION OF AAV-LIKE PARTICLES WITH
GENETIC MODIFICATIONS IN SPECIFIC CAPSID PROTEINS**

Modified Capsid Protein		Complementing Plasmid	
pVP0	(M1L; T138A; M203,211,235L)	pIM45	(WT)
pVP1	(T138A; M203,211,235L)	pVP2,3	(M1L)
pVP2	(M1L; M203,211,235L)	pVP1,3	(T138A)
pVP2A	(T138M)	pVP1,3	(T138A)

pVP3 (M1L; T138A)

pVP1,2 (M203,211,235L)

Capsid mutant complementation groups are co-transfected with pXX6 and pTRUF5 in 293 cells to produce particles.

5.3.2.4 AAV-LIKE PARTICLE FORMATION FROM CAPSID MUTANT CONSTRUCTS

The construction of plasmids that made only one or two of the capsid proteins allowed reexamination of the ability of various combinations of VP1, 2, and 3 to make viable AAV particles.

5.3.2.4.1 VP3 N-TERMINAL MUTATIONS

Since the mutation of the N- and C-terminal regions of VP3 has been reported to abolish AAV particle formation, the effects of the VP3 N-terminal M203L, M211L, and M235L mutations on particle formation were examined (FIG. 27A, Table 2). These mutations individually and combined in a pIM45 background (pM203L, pM211L, pM235L, and pM203,211,235L) were transfected into 293 cells with pXX6 and pTRUF5. Particles were purified from 293 cell lysates 72 hr post-transfection by iodixanol step gradients and equal volumes of the virus containing fraction were Western blotted and probed with the B1 antibody. While AAV particles were obtained from pM235L, the importance of the VP3 N-terminal region in particle assembly is illustrated by the fact that both the pM203L and pM211L mutant plasmids produced no particles (FIG. 27A). It was not clear whether this defect was due solely to mutation of the VP3 N-terminus, or because the M203L and M211L mutations were also present in the VP1 and VP2 proteins expressed from the pM203L and pM211L mutant plasmids.

5.3.2.5 MUTANTS EXPRESSING TWO CAPSIDS

To determine if any of the capsid proteins were non-essential for particle formation, the recovery of AAV-like particles lacking a specific capsid protein was examined. Constructs pVP2,3, pVP1,3, pVP1,2, and pVP1,2A were transfected individually into 293 cells in combination with pXX6 and pTRUF5 at equivalent molar ratios. Particles were purified from 293 cell lysates 72 hr post-transfection by iodixanol step gradients and equivalent volumes of the vector preparations were Western blotted and probed with B1 antibody (FIG. 27B). Particles were titered as described previously (Table 7, FIG. 30B).

As expected, AAV-like particles composed of VP2 and VP3 were obtained following transfection of pVP2,3. Due to the lack of the capsid sequences unique to VP1,

these particles displayed the *lip* phenotype with a particle to infectivity ratio approximately 3 logs lower than wild type (Table 7). This has been shown previously (Girod *et al.*, 1999; Hermonat *et al.*, 1984; Tratschin *et al.*, 1984; Wu *et al.*, 2000) and is presumably due to the absence of the VP1 phospholipase A activity. Surprisingly, an AAV-like particle formed in the absence of the previously reported critical VP2 capsid protein (Hoque *et al.*, 1999; Muralidhar *et al.*, 1994; Ruffing *et al.*, 1992) when VP1 and VP3 were present (FIG. 27B, pVP1,3 lane). Furthermore, these VP2 negative particles had virtually the same properties and yield as wild type particles (Table 7). Finally, the constructs that made only VP1 and VP2 (pVP1,2 and pVP1,2A) were unable to assemble a particle in the absence of VP3, irrespective of the level of VP2 expression (FIG. 27B, Table 7).

5.3.2.5.1 MUTANTS EXPRESSING A SINGLE CAPSID PROTEIN

The ability of a single capsid protein to form an AAV-like particle was tested next. Constructs pVP1, pVP2, pVP2A, and pVP3 were transfected individually into 293 cells in combination with pXX6 and pTRUF5 in equivalent molar ratios. As before, particles were purified from 293 cell lysates 72 hr post-transfection and equivalent volumes of the vector preps were Western blotted and probed with B1 antibody. Since the expression of VP1 and VP2 together did not form particles (see above), the formation of particles from them individually was not anticipated. While no particles formed in the presence of the two less abundant capsid proteins, an AAV-like particle composed of VP3 alone was readily obtained (FIG. 27C and Table 7). This result was in agreement with a previous insertional mutagenesis study, which also suggested that particles could form with VP3 alone (Rabinowitz *et al.*, 1999).

5.3.2.6 RECOMBINANT AAV PRODUCTION SYSTEM USING COMPLEMENTARY CAPSID PROTEIN MUTANTS

Since direct insertion of larger peptides after residue 138 leads to loss of VP3 expression, it was hypothesized that significant modification of VP1 and VP2 at residue 138 would require that wild type VP3 be provided *in trans* for efficient AAV production. The ability to complement a missing capsid protein by using the combination of plasmids described above and summarized in Table 8 was, therefore, tested, which express one and various combinations of two capsid proteins. To control for twice the Rep expression resulting from combining two pIM45-based plasmids that are used in this approach, a

construct, pVP0, was generated that eliminates expression of all of the capsid proteins with the mutations M1L, T138A, M203L, M211L, and M235L (Table 8). The capsid protein complementation groups include: pIM45 + pVP0, which makes wild type capsid proteins; constructs pVP1 + pVP2,3, which allows for exclusive modification of VP1; constructs pVP2 + pVP1,3, which allows for exclusive modification of the VP2; constructs pVP2A + pVP1,3, which allows for exclusive modification of and significant over-expression of VP2; and constructs pVP3 + pVP1,2 which allows for exclusive modification of VP3. As before, these groups were transfected into 293 cells (in combination with pXX6 and pTRUF5 at equivalent molar ratios), and particles were purified from 293 cell lysates 72 hr post-transfection by iodixanol step gradients and heparin column chromatography. Equivalent volumes of the vector preps were Western blotted and probed with B1 antibody (FIG. 28A), and titered as described above (Table 7).

Regardless of the complementation group employed, particles containing all three capsid proteins were recovered using this recombinant AAV production system. Interestingly, it was also observed that over-expression of VP2 resulted in the recovery of a particle in which VP2 is over-represented (FIG. 28A, pVP2A + pVP1,3). These particles contained lower amounts of VP1 and VP3, and VP2 levels that were nearly equivalent to VP3. (The slightly lower infectivity of the VP2A containing particle (Table 7) might be a reflection of the lower amounts of VP1 in these particles but this was not further explored.) All of the complementation groups produced virus yields and particle to infectivity ratios that were within a log of wild type virus. This was interpreted to mean that it could now be attempted to individually modify specific capsid proteins in regions of overlap (*e.g.*, residue 138). It was also noted that the mutations M203L and M211L, which are present in VP1 and VP2 when synthesized from pVP1,2 (Table 8), have little if any effect on the function of VP1 and VP2 in particle formation, when complemented with a wild type VP3 synthesized from pVP3 (Table 7). Thus, the effect of these mutations in the context of pIM45 (Table 7, mutants M203L and M211L) appeared to be entirely due to loss of VP3 function.

5.3.2.7 AAV-LIKE PARTICLES WITH FKN OR LEP INSERTED INTO VP1 AND VP2

Because direct insertion of large peptides after residue 138 resulted in the loss of VP3 expression, and the complementary capsid protein groups produced viable rAAV

particles, the ability to produce AAV-like particles with larger peptides inserted after residue 138 either simultaneously in VP1 and VP2 or exclusively in VP2 was next tested (FIG. 29A). Constructs that contained insertions in both VP1 and VP2 were complemented with pVP3, while those with insertions only in VP2 were complemented with pVP1,3. To make ligand insertion easier, *EagI/MluI* cloning sites were again inserted after amino acid position 138 in pVP1,2A and pVP2A as described earlier for pIM45 to create the plasmids pVP1,2AE/M138 and pVP2A-E/M. The VP2 over-expressing background was chosen to increase the incorporation of VP2-ligand fusion proteins into viral particles. Both the FKN and LEP coding sequences were inserted into pVP1,2A-E/M138 and pVP2A-E/M138 to make pVP1,2A-FKN, pVP2A-FKN, pVP1,2A-LEP, and pVP2A-LEP (FIG. 29A, FIG. 29B, FIG. 29C, Table 7). These plasmids were transfected into 293 cells in combination with pVP3 or pVP1,3, and pXX6 and pTRUF5 at equivalent molar ratios, and the resulting virus particles were purified with iodixanol step gradients. Equivalent volumes of the various preparations were then Western blotted in duplicate and probed with B1 or ligand-specific antibodies (anti-FKN or anti-LEP; FIG. 29B and FIG. 29C). In all cases, novel AAV-like particles were obtained in which the inserted sequences were present in VP1 and VP2, or just VP2. This was illustrated by an increase in the size of the VP1 and VP2 capsid proteins in blots probed with B1 antibody and confirmed with the ligand specific (FKN or LEP) antibodies. These iodixanol fractions were then titrated as described above (Table 7, FIG. 30B).

5.3.2.8 CHARACTERIZATION OF AAV-LIKE PARTICLES

To characterize the novel particles described in this study further, a portion of all of the virus stocks described above that were either missing a capsid protein or contained a modified capsid were purified by heparin column chromatography. Subsequently, approximately 10^{11} particles were Western blotted and probed with B1 antibody (FIG. 30A) to compare the stoichiometry of the capsid proteins in the various particles. Generally, the level of individual capsid proteins was similar to wild type with the following exceptions. First, as shown earlier, (FIG. 25A, FIG. 25B, FIG. 25C, FIG. 26, FIG. 28A, FIG. 28B) over-expression of VP2 (VP2A) leads to an altered capsid ratio in a particle composed of VP2 and VP3 (FIG. 30A, lane VP2A + VP3). This was true even when peptides of 76 (FKN) or 146 (LEP) amino acids were inserted after amino acid 138 of VP2A (compare

FIG. 30A, lanes pIM45 and VP2,3 with FKN or LEP inserted particles). Additionally, the relative amount of VP1-ligand fusion protein (and often wild type VP3) was reduced in these particles. Finally, the fact that the particles with FKN and LEP inserted in VP1 and VP2 could be purified by heparin chromatography suggested that ligands up to 18 kDa may not affect binding to heparan sulfate proteoglycan when inserted after residue 138.

To determine the relative ability of the novel particles to assemble, package DNA and infect cells, the particles were titered by the A20 ELISA assay (to estimate the total particles, empty and full), the real-time PCR[™] assay (to determine the titer of genome containing DNase resistant full particles), and the fluorescent cell assay (to determine the infectious particle titer). These assays were all performed on the iodixanol purified stocks (Table 7) and then the log relative infectivity was calculated (FIG. 30B).

With the exception of the mutants discussed earlier, all of the virus stocks contained A20 particle titers that were similar to wild type (Table 7, approximately $2-8 \times 10^{12}$ /ml). This was also true of the particles that contained a FKN or LEP insertion in VP1 and VP2 or in VP2 alone. Thus, the FKN and LEP insertions, and even a larger GFP insertion (discussed below), did not seem to affect viral assembly as judged by the conformation dependent A20 antibody (Table 7). When the relative packaging efficiency of the rAAV-like particles containing FKN or LEP ligands was examined (Table 7), the analysis revealed these particles package DNA nearly as well as wild type, within 1 log (Table 7, genomes/ml). A striking difference, however, was noticed when the FKN and LEP particles were tested for infectivity. Particles that contained FKN and LEP insertions only in VP2 had particle to infectivity ratios that were essentially the same as wild type (Table 7 and FIG. 30B, compare pIM45-E/M 138, pIM45 and VP1,3 with VP2AFKN + VP1,3 and VP2A-LEP + VP1,3). However, particles that had a FKN or LEP insertion in both VP1 and VP2 were 4-5 logs less infectious. The loss in infectivity was comparable to that seen with all particles that had wild type AAV capsid proteins but were missing VP1 (Table 7, FIG. 30B, lanes pVP2,3; pVP2A,3 and VP3). Thus, it appeared that if the foreign ligand was inserted exclusively into the N-terminus of the non-essential VP2 capsid, a ligand as large as 138 amino acids could be tolerated with minimal loss of packaging efficiency or infectivity.

5.3.2.9 AAV-LIKE PARTICLES WITH GFP INSERTED INTO VP1 AND VP2

Since FKN and LEP had little effect on overall vector yields, it needed to be determined if insertions significantly larger than the VP1 unique region (137 residues) are still able to form particles. Therefore, the coding sequence for the 30 kDa GFP protein (238 residues) was inserted into pVP1,2A-E/M138 and pVP2A-E/M138 for complementation with pVP3 and pVP1,3 respectively. These particles were purified using iodixanol step gradient followed by heparin chromatography, and titered as described above (FIG. 30B, Table 7). Western blot analysis of equal volumes revealed that both VP1 and VP2 had the expected increased molecular weight due to the insertion of GFP (FIG. 30C). While this experiment was primarily meant to be a test of the size limit for insertions after residue 138, the development of a fluorescently tagged vector was also a potentially interesting tool for studying the cellular entry and trafficking of recombinant AAV particles. As with the FKN and LEP insertions, insertion of the GFP sequence into both VP1 and VP2 was much less successful than insertion into VP2 alone. While the yield of particles obtained with GFP inserted into both VP1 and VP2 appeared to be similar to wild type (FIG. 30C and Table 6), these vectors had a more severe defect in packaging (Table 7, almost 2 logs down) and were severely defective for infectivity (Table 7 and FIG. 30B, approximately 5 logs). In contrast, GFP insertions into VP2A alone produced stocks that were 3-4 logs down for infectivity (Table 7 and FIG. 30B).

To determine if the particles that contained GFP inserts in both VP1 and VP2 (VP1,2A-GFP+VP3) behaved normally with respect to entry and trafficking, confocal microscopy was used. Confocal microscopic analysis of these particles in the absence (FIG. 31, top panel) and presence (FIG. 8, bottom panel) of helper Ad 5 infection revealed that, in the absence of helper virus, these AAV-like particles slowly accumulate in endosomes and/or cytoplasm peri-nuclearly over a 24 hr period. However, dramatic changes were observed when helper virus was present, with the appearance of the viral GFP signal within the nucleus as early as 1 hr. These results were in agreement with a previous report on the facilitation of AAV trafficking by adenovirus (Xiao *et al.*, 2002). Thus, the particles containing a 30 kDa GFP insertion in VP1 and VP2 behaved essentially like wild type virus with respect to infection and trafficking in response to Ad coinfection.

5.3.3 DISCUSSION

The AAV particle is capable of transducing a wide range of dividing and non-dividing cell types. The promiscuity of this gene therapy vector is due in part to the widespread distribution of its primary receptors (Kern *et al.*, 2003; Opie *et al.*, 2003; Qing *et al.*, 1999; Summerford *et al.*, 1999; Summerford and Samulski, 1998) and the strong electrostatic interaction between cell surface heparan sulfate and the spike protrusion at the particle's three-fold axes (Kern *et al.*, 2003; Opie *et al.*, 2003; Summerford and Samulski, 1998). To date, most of the strategies for retargeting AAV have involved inserting short, linear targeting sequences directly into the capsid genes, normally VP3, which is the most abundant capsid protein (Buning *et al.*, 2003). The major goal of the present study was to see if it was possible to incorporate significantly larger peptides into the AAV particle as a first step in retargeting the vector to alternative receptors requiring conformation-dependent ligands. Based on the symmetry of the particle and capsid protein molecular weight estimates (Xie *et al.*, 2002), it has been proposed that of the 60 capsid proteins that make up a given particle, approximately 3 are VP1, 3 are VP2, and 54 are VP3. Thus, depending on the position within the *cap* ORF, retargeting sequences can result in the incorporation of differing numbers of ligands per particle. For instance, insertions immediately after residue 138 in the VP1/VP2 region have been shown to expand the tropism of the virus (Shi *et al.*, 2001; Wu *et al.*, 2000) following the incorporation of approximately 6 modified capsid proteins (3 VP1 and 3 VP2).

Theoretically the insertion of a single full length ligand could retarget the particle to a receptor, binding its ligand with 1:1 stoichiometry. Therefore, insertions to residue 138 were confined to minimize disruption of the overall structural features of the particle (as 60 large ligands seemed excessive and more likely to sterically hinder assembly than 6 ligands). However, direct insertion of the coding sequence for FKN and LEP at this position led to the loss of VP3 expression (FIG. 24A), and did not result in particle formation (FIG. 24B). This was seen as well by others (Rabinowitz *et al.*, 1999) and was presumably due to disruption of the read through translational initiation required for production of the critical VP3 protein (Becerra *et al.*, 1988). It was necessary, therefore, to consider the alternative of using insertions in only one capsid protein at a time with the other two being functionally wild type. To test this possibility, a series of complementing

plasmids was constructed (Table 8) that would allow insertions into only one of the three capsid proteins at a time.

5.3.3.1 VP3-LIKE PROTEINS CAN BE TRANSLATED FROM 3 DIFFERENT METHIONINE CODONS AND THE FIRST 8 AMINO ACIDS OF VP3 APPEAR TO BE ESSENTIAL FOR VP3 CAPSID ASSEMBLY

While VP1 and VP2 synthesis were easily eliminated by mutation of their respective start codons (FIG. 27B), the elimination of VP3 *per se* was interesting, requiring multiple mutations to generate the construct pVP1,2 (FIG. 25B). Ruffing *et al.* (1992) had also previously seen alternative VP3-like proteins when the start codon was changed to leu. Here, it has been demonstrated that the alternative VP3 species are due to the use of alternative start codons downstream of the normal ATG for VP3 (M203). Read-through translational initiation on the 2.3 kb mRNA continued for an additional 32 amino acids after M203 to positions M211 and M235. Since the M203L or M211L mutations prevented particle recovery (FIG. 27A), it appears that these residues play critical roles in particle assembly and/or stability. M203L results in an N-terminal truncation of VP3 (VP3a), while M211L is a point mutation in full length VP3. These mutations are present in all three capsid proteins, but appear to be critical to VP3 as the combination of pVP1,2 + pVP3 produced essentially wild type recombinant particles. The formation of particles from the complementation groups are examples of positional rescue of mutations at the VP3 N-terminus, as the M203L and M211L mutations that are required to eliminate VP3 expression (FIG. 25B) abolish particle formation (FIG. 26A) when present in all three capsid proteins, yet yield particles that are essentially wild type when these mutations are present only in VP1 and/or VP2 (FIG. 28A and FIG. 28B). The design of this production system results in the VP3 protein never having the M203,211,235L mutations (Table 8). In contrast, manipulation of the common C-terminus of the *cap* ORF is apparently different (Ruffing *et al.*, 1994; Wu *et al.*, 2000). A recent example of positional rescue was reported for the insertion of a 6xHis tag (for recombinant vector purification purposes) at the extreme C-terminus of the *cap* ORF (Zhang *et al.*, 2002c). In this report, the VP1 and VP2 capsid proteins were shown to be responsible for the defects in particle formation when the insertion was present in all three capsid proteins, and this position was rescued when the tag was present only in VP3.

5.3.3.2 VP2 APPEARS REDUNDANT AND NON-ESSENTIAL FOR VIRAL INFECTIVITY

Surprisingly, the AAV-like particle composed of only VP1 and VP3 had infectious titers within a factor of 4 of wild type (FIG. 27B and FIG. 30B, Table 6), and particle to infectivity ratios which were identical to wild type. Thus, VP2 appeared to be a redundant capsid that is not essential for infectivity. This made it an ideal candidate for the insertion of large peptides for the purpose of retargeting the particle.

Earlier work had reported the identical *cap* mutant to be defective for production of infectious virus (Muralidhar *et al.*, 1994). At present, no satisfactory explanation exists for this discrepancy. One can only speculate that improvement in AAV production and purification may have allowed characterization of this particle, or that there might have been additional cryptic mutations in the earlier constructs. Similarly, expression of the three capsid proteins in a baculovirus system also suggested that VP2 may play a role in particle assembly (Ruffing *et al.*, 1992; Steinbach *et al.*, 1997). Thus, the isolation of AAV-like particles from pVP1,3 was unexpected, since critical aspects of nuclear localization (Hoque *et al.*, 1999; Ruffing *et al.*, 1992) and particle formation (Ruffing *et al.*, 1992) have been attributed to VP2. In contrast to that work, attempts by the inventors to make VP3 only or VP2 negative particles have been consistently in the presence of AAV replication proteins, rAAV DNA, and Ad helper functions. This may partly explain the discrepancy with the baculovirus systems and earlier experiments in Cos cells. Alternatively, this may reflect a property of AAV assembly in these cell types.

Curiously, while VP2 negative particles (VP1,3) appear to be functionally wild type, the VP2A + VP3 group or VP3 alone produce particles that are more defective than those that are missing only VP1 (VP2,3) (FIG. 30B). Thus, in the absence of VP1, VP2 may perform some function in AAV infection. A comparison of the characteristics of the VP3 particle with the VP2,3 particle (FIG. 30B, Table 7) suggests that the additional VP2 residues may facilitate transduction in the absence of VP1. Possibly, the basic residues that cluster in the VP2 N-terminal extension of VP3 which are capable of being nuclear localization signals (Hoque *et al.*, 1999) play a role. However, the VP2A,3 particle is less infectious than VP2,3 showing that the inclusion of more VP2 unique sequence into the particle is detrimental (FIG. 30B, Table 7).

5.3.3.3 VP3 IS THE ONLY CAPSID PROTEIN REQUIRED TO FORM GENOME CONTAINING PARTICLES

AAV-like particles were obtained from any combination of capsid proteins or capsid mutants as long as VP3 was present (FIG. 30A and FIG. 30C, Table 8). Furthermore, VP3 alone was sufficient to make viral particles. Viral particles composed of VP2,3, VP1,3, and VP3 were obtained only at wild type particle titers (both full and empty) (FIG. 27B, FIG. 27C, FIG. 28, FIG. 30A and Table 7). As expected, particles that were missing VP1 (VP2,3, VP2A,3 and VP3) were severely defective for infectivity (FIG. 30B, Table 7). This defect is presumably due to the absence of the phospholipase activity in the N-terminal region of VP1 as previously described (Girod *et al.*, 2002; Hermonat *et al.*, 1984; Tratschin *et al.*, 1984; Wu *et al.* 2000).

The recovery of the VP3 only particle (FIG. 27C and FIG. 30A, Table 2) agrees with a previous insertional mutagenesis study in which a particle was isolated that appeared to be composed exclusively of VP3 (Rabinowitz *et al.*, 1999). Taken together, these results show that neither VP1 nor VP2 is absolutely required for nuclear localization of VP3 (Ruffing *et al.*, 1992; Wistuba *et al.*, 1997) and begs the question as to which nuclear localization signals are employed by the three capsid proteins.

5.3.3.4 COMPLEMENTARY CAPSID PROTEIN EXPRESSION ALLOWS FORMATION OF PARTICLES WITH LARGE INSERTIONS EXCLUSIVELY IN VP2 THAT HAVE ONLY MODEST DEFECTS IN VIRAL INFECTIVITY

The key finding in this study is that it is possible to insert substantially larger peptides into AAV capsid proteins than previously shown provided that the foreign peptide is fused to only one of the three capsid proteins. In initial studies, focus primarily has been on insertions into the minor capsid proteins. The insertion of FKN and LEP simultaneously into VP1 and VP2 had little effect on packaging efficiency, but resulted in particles with low infectious titers (FIG. 30B, Table 7). This may be partly explained by spatial distortion of the phospholipase A2 motifs, but defects in viral uncoating cannot be ruled out. To rescue position 138 for insertion of large peptides with respect to infectivity, the inserted peptide had to be confined to VP2 exclusively. These AAV-like particles were within a log of wild type particle and infectious titers and had particle to infectivity ratios virtually identical to wild type virus (FIG. 30B, Table 7). Thus, ligands as large as 146 amino acids (LEP) appear to be readily accommodated by this method. In contrast, when the 238 amino acid GFP protein was inserted into VP2, there was a significant drop in the

particle to infectivity ratio (FIG. 30B, Table 7). It may be possible to correct this by increasing the intracellular expression of VP1, which was severely under-represented in the VP2A-GFP + VP3 particles, or decreasing the level of the VP2 ligand concentration. This is currently being explored.

Nevertheless, it was possible to obtain and visualize particles with GFP inserted into both VP1 and VP2 (FIG. 30C and FIG. 31) and these VP1,2A-GFP + VP3 particles appeared to traffic in a fashion similar to that described previously for wild type virus (Xiao *et al.*, 2002), suggesting that insertions as large as the 30 kDa GFP protein could be tolerated. Ligand insertions have not yet found that were exclusively in VP1 or VP3 at any surface positions previously shown to accommodate shorter peptides (Girod *et al.*, 2002; Shi *et al.*, 2003; Wu *et al.*, 2000). However, it may be that these positions are useful for insertion of larger ligands with the use of the separate capsid expression plasmids described here.

In summary, while VP3 alone is sufficient to form a particle capable of protecting the viral genome and VP1 is required for efficient viral infectivity, VP2 is non-essential and tolerates large peptide insertions at its N-terminus. The stoichiometry of the particle can be altered if VP2 is significantly over-expressed in the presence of native levels of VP1 and VP3. While the inserted sequences studied here are themselves potential targeting ligands, this system could also be applied to the insertion of large conjugate-based linker sequences (Ponnazhagan *et al.*, 2002; Ried *et al.*, 2002) or for the presentation of large immunogenic peptides for vaccine development using empty particles formed with this system as the platform for epitope presentation. Future work with the described FKN and LEP particles will involve testing their ability to bind their respective receptors. The GFP containing particles may have potential use in real time *in vivo* fluorescent monitoring of events that occur during infection. It is evident that optimal retargeting of these particles with insertions at the N-terminus of VP2 may require manipulation of linker sequences between the inserted ligand and VP2 to optimize presentation of the ligand binding domain. Furthermore, mutation of the recently identified residues involved in binding heparan sulfate proteoglycan (Kern *et al.*, 2003; Opie *et al.*, 2003) will also be required to restrict these vectors to cellular entry via the targeting ligand/receptor interaction. Importantly, the system described here for modifying capsid proteins with larger peptide insertions in specific capsid proteins should facilitate development of retargeted AAV vectors for

clinically relevant cell types and be applicable to all AAV serotypes and chimeric type particles (Bowles *et al.*, 2003; Gao *et al.*, 2003; Hauck *et al.*, 2003; Hildinger *et al.*, 2001; Rabinowitz *et al.*, 2002).

6.0 REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

CLAIMS:

1. A recombinant adeno-associated viral expression system that expresses at least a first, second, and third distinct capsid proteins, comprising:
 - (a) a first expression vector that encodes a Vp3 capsid protein; and
 - (b) a second expression vector that encodes a Vp1 capsid protein and a modified Vp2 capsid protein.
2. The recombinant adeno-associated viral expression system according to claim 1, wherein said Vp3 capsid protein is translated from methionine codon M203, M211, or M235.
3. The recombinant adeno-associated viral expression system according to claim 1 or claim 2, wherein said Vp3 capsid protein is produced at or near wild-type levels.
4. The recombinant adeno-associated viral expression system according to any preceding claim, wherein said Vp1 capsid protein is produced at or near wild-type levels.

5. The recombinant adeno-associated viral expression system according to any preceding claim, wherein said modified Vp2 capsid protein comprises at least a first mutation.
6. The recombinant adeno-associated viral expression system according to any preceding claim, wherein said modified Vp2 capsid protein comprises at least a first insertion mutation, point mutation, frame-shift mutation, or deletion mutation.
7. The recombinant adeno-associated viral expression system according to any preceding claim, wherein said modified Vp2 capsid protein comprises at least a first insertion mutation, point mutation, frame-shift mutation, or deletion mutation that substantially reduces Vp2 capsid protein production when compared to wild-type.
8. The recombinant adeno-associated viral expression system according to any preceding claim, wherein said modified Vp2 capsid protein comprises at least a first insertion mutation, point mutation, frame-shift mutation, or deletion mutation that essentially eliminates Vp2 capsid protein production when compared to wild-type.
9. The recombinant adeno-associated viral expression system according to any preceding claim, wherein said modified Vp2 capsid protein comprises at least a first

insertion mutation, point mutation, frame-shift mutation, or deletion mutation that eliminates Vp2 capsid protein production when compared to wild-type.

10. The recombinant adeno-associated viral expression system according to any one of claims 5 to 9, wherein said at least a first mutation comprises an insertion of at least a first peptide or protein targeting ligand.
11. The recombinant adeno-associated viral expression system according to any one of claims 5 to 10, wherein said at least a first mutation alters, impairs, or prevents the binding of said capsid protein to a mammalian cell surface receptor or binding site.
12. The recombinant adeno-associated viral expression system according to any one of claims 5 to 11, wherein said at least a first mutation is a deletion or a frame-shift that alters or eliminates at least a first amino acid residue required for binding of said capsid protein to a mammalian cell surface receptor or binding site.
13. The recombinant adeno-associated viral expression system according to any one of claims 5 to 12, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein of less than about 40 kDa.

14. The recombinant adeno-associated viral expression system according to any one of claims 5 to 13, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein of less than about 30 kDa.
15. The recombinant adeno-associated viral expression system according to any one of claims 5 to 14, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein of less than about 20 kDa.
16. The recombinant adeno-associated viral expression system according to any one of claims 5 to 15, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein of less than about 10 kDa.
17. The recombinant adeno-associated viral expression system according to any one of claims 5 to 12, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein ligand of about 5 kDa to about 45 kDa.
18. The recombinant adeno-associated viral expression system according to claim 17, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein ligand of about 10 kDa to about 40 kDa.

19. The recombinant adeno-associated viral expression system according to claim 18, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein ligand of about 15 kDa to about 35 kDa.
20. The recombinant adeno-associated viral expression system according to claim 19, wherein said at least a first insertion mutation comprises a nucleic acid sequence that encodes a protein ligand of about 20 kDa to about 30 kDa.
21. The recombinant adeno-associated viral expression system according to any one of claims 5 to 20, wherein said at least a first insertion mutation occurs at amino acid position 138, amino acid position 139, amino acid position 140, or amino acid position 141.
22. The recombinant adeno-associated viral expression system according to any preceding claim, further comprising a third expression vector that encodes the adenoviral helper gene products to permit production of said expression system in an adenovirus-free environment.
23. The recombinant adeno-associated viral expression system according to any preceding claim, further comprising a fourth expression vector that comprises an expression cassette flanked by AAV2 terminal repeat sequences.

24. The recombinant adeno-associated viral expression system according to claim 23, wherein said expression cassette comprises a first polynucleotide that comprises, consists essentially of, or consists of, a first nucleic acid segment that encodes at least a first therapeutic agent.
25. The recombinant adeno-associated viral expression system according to claim 24, wherein said therapeutic agent is a peptide, polypeptide, protein, catalytic RNA molecule, ribozyme, or an antisense oligonucleotide or antisense polynucleotide.
26. The recombinant adeno-associated viral expression system according to claim 24, wherein said first polynucleotide further comprises a second nucleic acid segment that comprises, consists essentially of, or consists of, a heterologous, tissue-specific, constitutive or inducible promoter operably linked to said first nucleic acid segment, wherein said promoter expresses said therapeutic agent.
27. The recombinant adeno-associated viral expression system according to claim 26, wherein said promoter is selected from the group consisting of a CMV promoter, a β -actin promoter, an insulin promoter, an enolase promoter, a BDNF promoter, an NGF promoter, an EGF promoter, a growth factor promoter, an axon-specific promoter, a dendrite-specific promoter, a brain-specific promoter, a hippocampal-specific

promoter, a kidney-specific promoter, an elafin promoter, a cytokine promoter, an interferon promoter, a growth factor promoter, an alpha-1 antitrypsin promoter, a brain-specific promoter, a neural cell-specific promoter, a central nervous system cell-specific promoter, a peripheral nervous system cell-specific promoter, an interleukin promoter, a serpin promoter, a hybrid CMV promoter, a hybrid β -actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

28. The recombinant adeno-associated viral expression system according to claim 27, wherein said promoter is a mammalian β -actin promoter.
29. The recombinant adeno-associated viral expression system according to claim 24, wherein said first polynucleotide further comprises a third nucleic acid segment that comprises, consists essentially of, or consists of, an enhancer sequence operably linked to said first nucleic acid segment and said second nucleic acid segment.
30. The recombinant adeno-associated viral expression system according to claim 29, wherein said enhancer sequence comprises a CMV enhancer, a synthetic enhancer, a liver-specific enhancer, an vascular-specific enhancer, a brain-specific enhancer, a neural cell-specific enhancer, a lung-specific enhancer, a muscle-specific enhancer, a kidney-specific enhancer, a pancreas-specific enhancer, or an islet cell-specific enhancer.

31. The recombinant adeno-associated viral expression system according to claim 30, wherein said enhancer sequence comprises a CMV enhancer.
32. The recombinant adeno-associated viral expression system according to claim 24, wherein said first nucleic acid segment further comprises a post-transcriptional regulatory sequence or a polyadenylation signal.
33. The recombinant adeno-associated viral expression system according to claim 32, wherein said regulatory sequence comprises a woodchuck hepatitis virus post-transcription regulatory element, or said polyadenylation signal comprises a bovine growth hormone gene polyadenylation signal.
34. The recombinant adeno-associated viral expression system according to any one of claims 24 to 34, wherein said at least a first therapeutic agent is a peptide, protein, or polypeptide selected from the group consisting of an adrenergic agonist, an anti-apoptosis factor, an apoptosis inhibitor, a cytokine receptor, a cytokine, a cytotoxin, an erythropoietic agent, a glutamic acid decarboxylase, a glycoprotein, a growth factor, a growth factor receptor, a hormone, a hormone receptor, an interferon, an interleukin, an interleukin receptor, a kinase, a kinase inhibitor, a nerve growth factor, a netrin, a neuroactive peptide, a neuroactive peptide receptor, a neurogenic factor, a

neurogenic factor receptor, a neuropilin, a neurotrophic factor, a neurotrophin, a neurotrophin receptor, an N-methyl-D-aspartate antagonist, a plexin, a protease, a protease inhibitor, a protein decarboxylase, a protein kinase, a protein kinase inhibitor, a proteolytic protein, a proteolytic protein inhibitor, a semaphorin, a semaphorin receptor, a serotonin transport protein, a serotonin uptake inhibitor, a serotonin receptor, a serpin, a serpin receptor, and a tumor suppressor.

35. The recombinant adeno-associated viral expression system according to claim 34, wherein said polypeptide is selected from the group consisting of BDNF, CNTF, CSF, EGF, FGF, G-CSF, GM-CSF, gonadotropin, IFN, IFN-1, M-CSF, NGF, PDGF, PEDF, TGF, TGF- β 2, TNF, VEGF, prolactin, somatotropin, XIAP1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-10(187A), viral IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, and IL-18.
36. The recombinant adeno-associated viral expression system according to claim 35, wherein said polypeptide is selected from the group consisting of BDNF, CNTF, PEDF, TGF, TNF, VEGF, and XIAP1.
37. A recombinant adeno-associated viral vector comprising the recombinant adeno-associated viral expression system of any preceding claim.

38. The recombinant adeno-associated viral vector according to claim 37, wherein said vector is selected from the group consisting of AAV serotype 1, AAV serotype 2, AAV serotype 3, AAV serotype 4, AAV serotype 5, and AAV serotype 6.
39. A virion or viral particle for the transfection of mammalian cells, comprising the recombinant adeno-associated viral expression system of any one of claims 1 to 36, or the recombinant adeno-associated viral vector according to claim 37 or claim 38.
40. A plurality of infectious AAV particles, comprising the recombinant adeno-associated viral expression system of any one of claims 1 to 36, the recombinant adeno-associated viral vector according to claim 37 or claim 38, or the virion or viral particle according to claim 39.
41. An isolated host cell comprising:
- (a) the recombinant adeno-associated viral expression system of any one of claims 1 to 36;
 - (b) the recombinant adeno-associated viral vector according to claim 37 or claim 38;
 - (c) the virion or viral particle according to claim 39; or

- (d) the plurality of infectious AAV particles according to claim 40.
42. The isolated host cell according to claim 41, wherein said cell is a mammalian host cell.
43. The isolated host cell according to claim 41 or claim 42, wherein said cell is a human, primate, murine, feline, canine, porcine, ovine, bovine, equine, epine, caprine, or lupine cell.
44. The isolated host cell according to any one of claims 41 to 43, wherein said cell is a human endothelial, vascular, epithelial, liver, lung, heart, pancreas, kidney, muscle, bone, blood, neural, or brain cell.
45. A composition comprising:
- (a) the recombinant adeno-associated viral expression system of any one of claims 1 to 36;
- (b) the recombinant adeno-associated viral vector according to claim 37 or claim 38;
- (c) the virion or viral particle according to claim 39;

- (d) the plurality of infectious AAV particles according to claim 40; or
 - (e) the isolated host cell according to any one of claims 41 to 44.
46. The composition according to claim 45, further comprising a pharmaceutical excipient, buffer, or diluent.
47. The composition according to claim 45 or claim 46, further comprising a polymer, a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle.
48. The composition according to any one of claims 45 to 47, formulated for administration to a human.
49. The composition according to any one of claims 45 to 48, for use in therapy.
50. The composition according to any one of claims 45 to 49, for use in the therapy of cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, liver disease, neurological disease, neurosensory dysfunction,

stroke, heart attack, congenital myopathy, ischemia, pulmonary disease, circulatory disease, bone disease, hematological disease, neuromuscular disorders, eating disorders, neurological diseases, neuroskeletal impairment or disability, Alzheimer's disease, Huntington's disease, Graves' disease, or Parkinson's disease.

51. Use of a composition according to any one of claims 45 to 50, in the manufacture of a medicament for treating a disease or dysfunction in a mammal.
52. The use according to claim 51, in the manufacture of a medicament for treating a disease or dysfunction in a human.
53. The use according to claim 51 or claim 52, in the manufacture of a medicament for treating cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, liver disease, neurological disease, neurosensory dysfunction, stroke, ischemia, neuromuscular disorders, eating disorders, neurological diseases, neuroskeletal impairment or disability, Alzheimer's disease, Huntington's disease, Parkinson's disease, or pulmonary disease.
54. A kit comprising:

- (a) (i) the recombinant adeno-associated viral expression system of any one of claims 1 to 36;
 - (ii) the recombinant adeno-associated viral vector according to claim 37 or claim 38;
 - (iii) the virion or viral particle according to claim 39;
 - (iv) the plurality of infectious AAV particles according to claim 40;
 - (v) the isolated host cell according to any one of claims 41 to 44; or
 - (vi) The composition according to any one of claims 45 to 48;
- and
- (b) instructions for using said kit.

55. A method for targeting an AAV virion or viral particle to a mammalian cell that comprises a cell-surface receptor, said method comprising the step of: providing to a population of cells an AAV virion or viral particle that comprises the recombinant adeno-associated viral expression system of any one of claims 1 to 36, or the recombinant adeno-associated viral vector according to claim 37 or claim 38; in an amount and for a time effective to target said virion or said viral particle to cells of said population that express said cell-surface receptor.

56. A method for targeting an expressed therapeutic agent to a mammalian cell that comprises a cell-surface receptor, said method comprising the step of providing to a

mammal that comprises a population of said cells a biologically-effective amount of the recombinant adeno-associated viral expression system of any one of claims 1 to 36, or the recombinant adeno-associated viral vector according to claim 37 or claim 38.

57. The method according to claim 56, wherein said expressed therapeutic agent is a peptide ligand, a polypeptide, a protein, an antibody, an antigen binding fragment, a catalytic RNA molecule, or an antisense molecule.
58. A method for preventing, treating or ameliorating the symptoms of a disease, dysfunction, or deficiency in a mammal, said method comprising administering to said mammal the virion or viral particle according to claim 39, the plurality of infectious AAV particles of claim 40, or the composition according to any one of claims 45 to 50, in an amount and for a time sufficient to treat or ameliorate the symptoms of said disease, dysfunction, or deficiency in said mammal.
59. The method according to claim 58, wherein said mammal is a human.
60. The method according to claim 58 or claim 59, wherein said virion, said viral particle, said plurality of viral particles, or said composition, is administered to said mammal

intramuscularly, intravenously, subcutaneously, intrathecally, intraperitoneally, or by direct injection into an organ or a tissue of said mammal.

61. The method according to claim 60, wherein said organ or tissue is selected from the group consisting of bone, skin, pancreas, liver, heart, lung, brain, kidney, joint, and muscle.
62. A VP2-free rAAV expression system comprising:
 - (a) an rAAV vector that comprises at least a first heterologous nucleic acid segment inserted into the VP2 capsid-encoding sequence region, said segment encoding at least a first heterologous peptide; and
 - (b) at least a second expression vector that expresses functional VP3 capsid proteins substantially in the absence of VP2 protein.
63. The VP2-free rAAV expression system according to claim 62, wherein said rAAV vector substantially lacks VP1 expression and lacks VP2 expression when compared to wild-type.
64. The VP2-free rAAV expression system according to claim 62 or claim 63, wherein said at least a first heterologous peptide is expressed on the surface of an rAAV virion comprising said vector.

65. The VP2-free rAAV expression system according to any one of claims 62 to 64, wherein said at least a first peptide selectively targets said rAAV virion to at least a first host cell.
66. The VP2-free rAAV expression system according to any one of claims 62 to 65, wherein said rAAV vector further comprises a second nucleic acid segment that comprises, consists essentially of, or consists of a second exogenous polynucleotide operably positioned downstream and under the control of a promoter that expresses said second exogenous polynucleotide in a cell comprising said expression system.
67. The VP2-free rAAV expression system according to any one of claims 62 to 66, comprised within an infectious adeno-associated viral particle.
68. The VP2-free rAAV expression system according to any one of claims 62 to 67, comprised within a pharmaceutical vehicle.
69. An isolated mammalian host cell comprising the VP2-free rAAV expression system according to any one of claims 62 to 68.
70. The isolated mammalian host cell according to claim 69, wherein said host cell is a human host cell.

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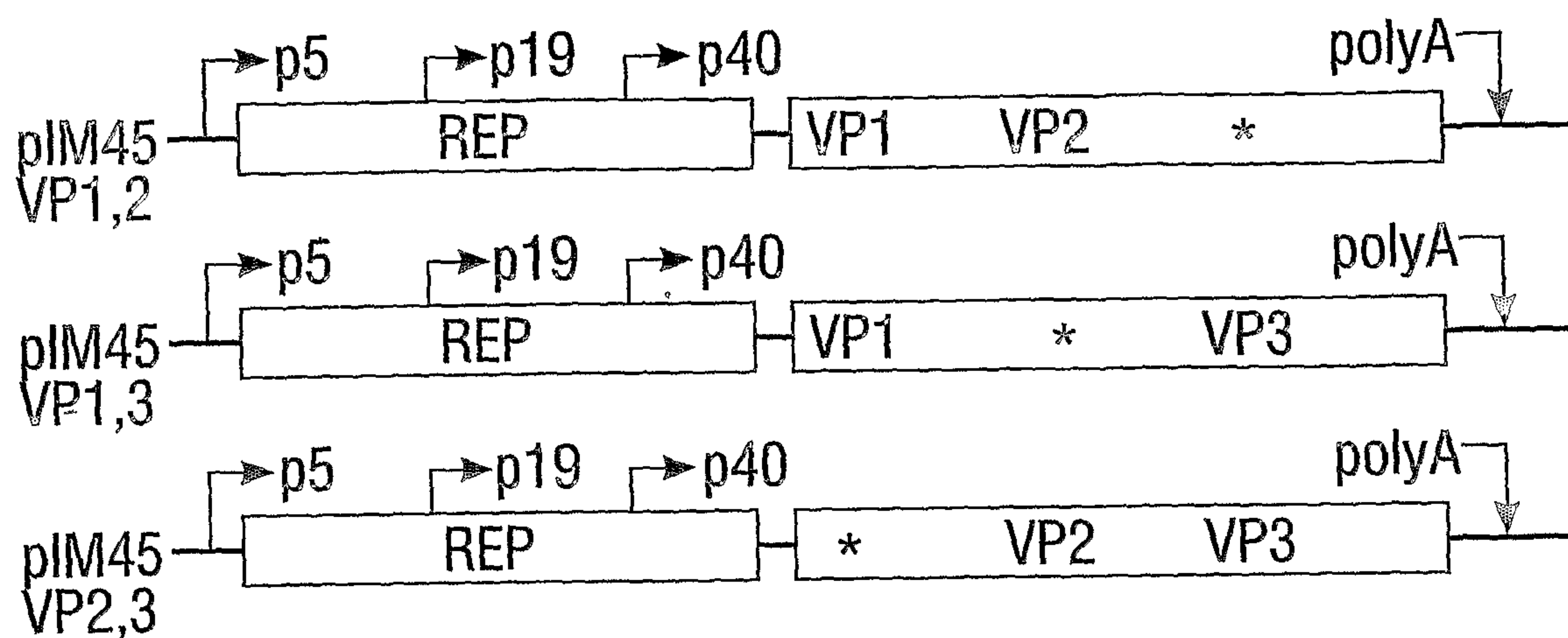


FIG. 1A-1

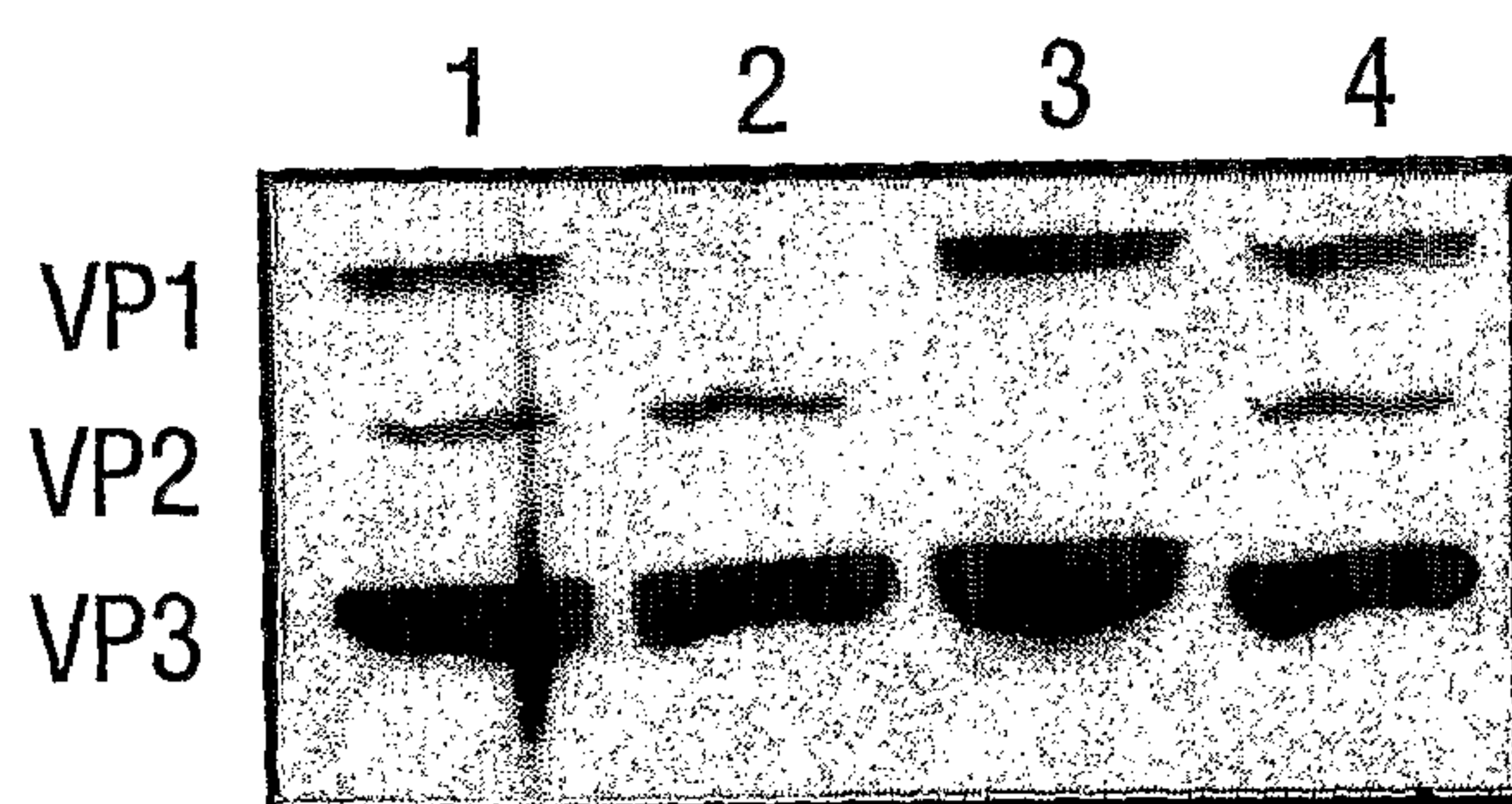


FIG. 1A-2

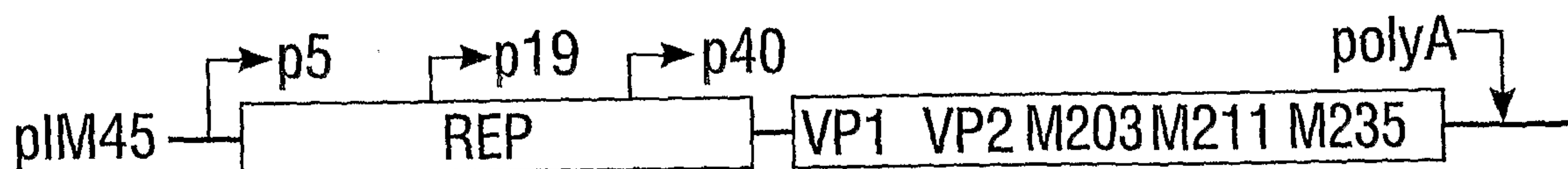


FIG. 1B-1

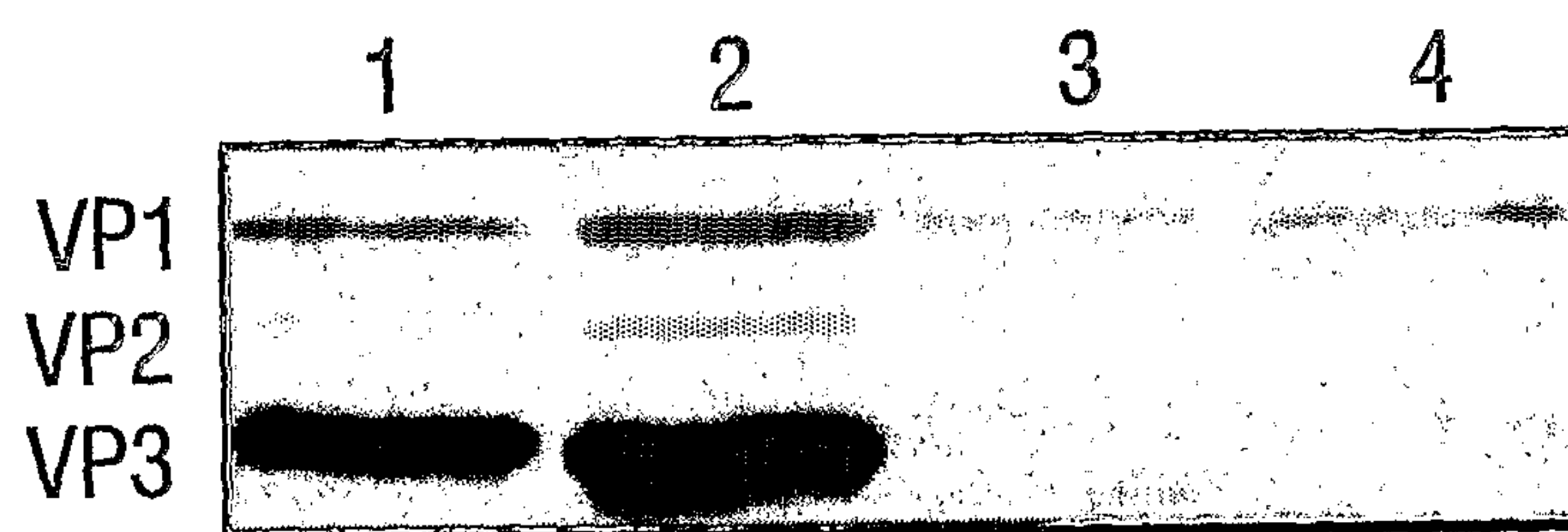


FIG. 1B-2

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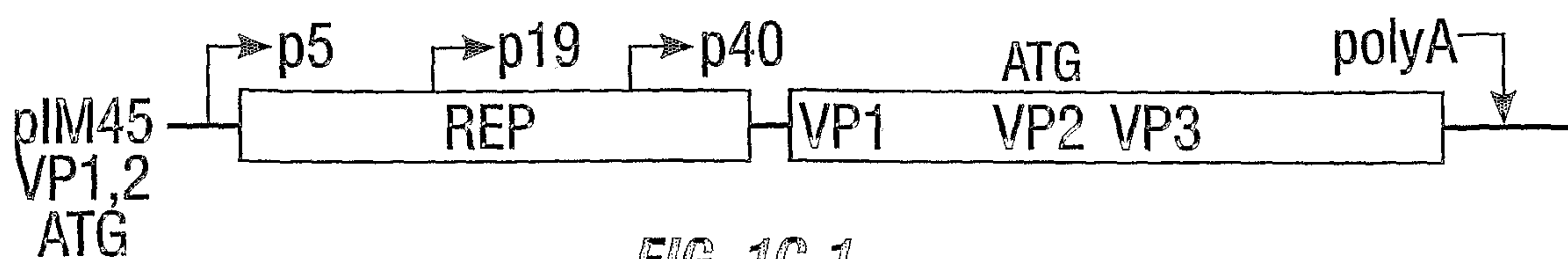


FIG. 1C-1

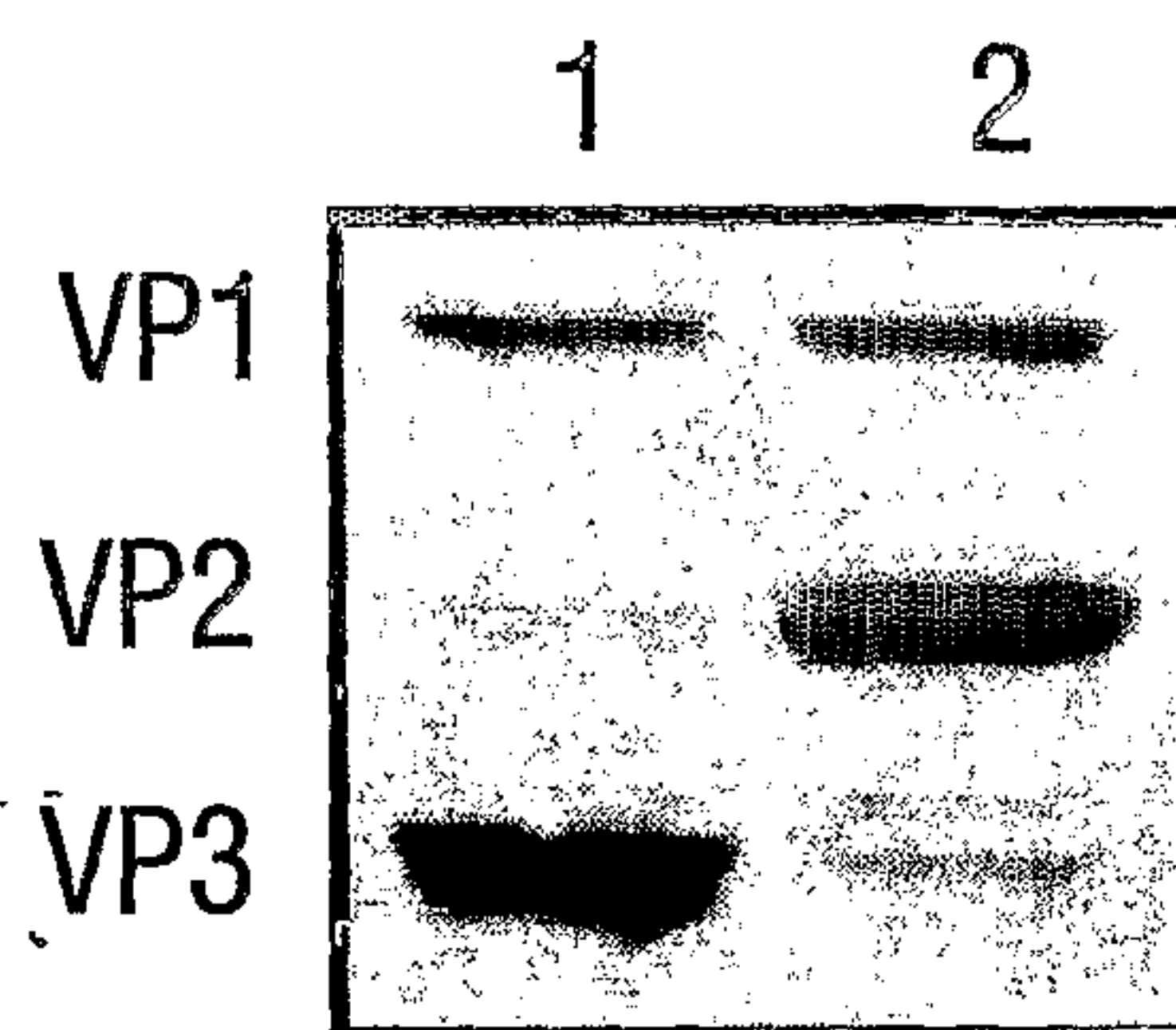


FIG. 1C-2

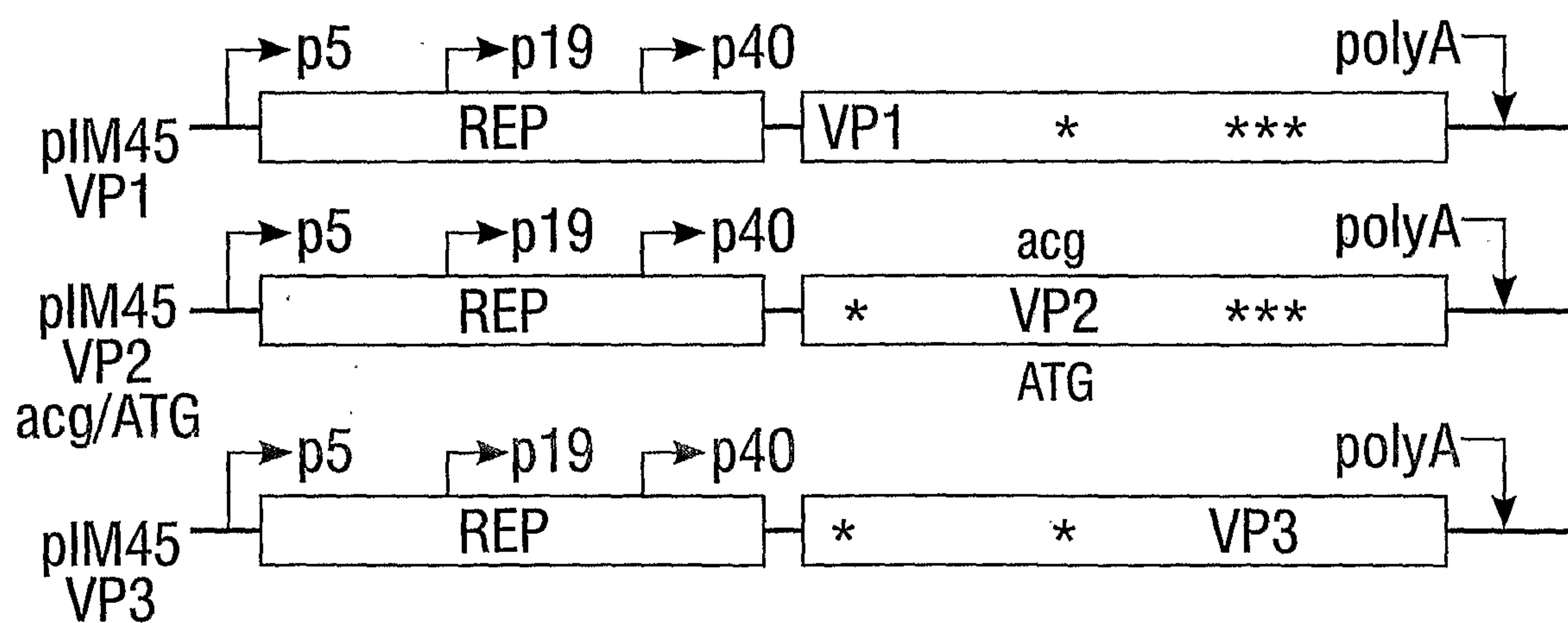


FIG. 2A

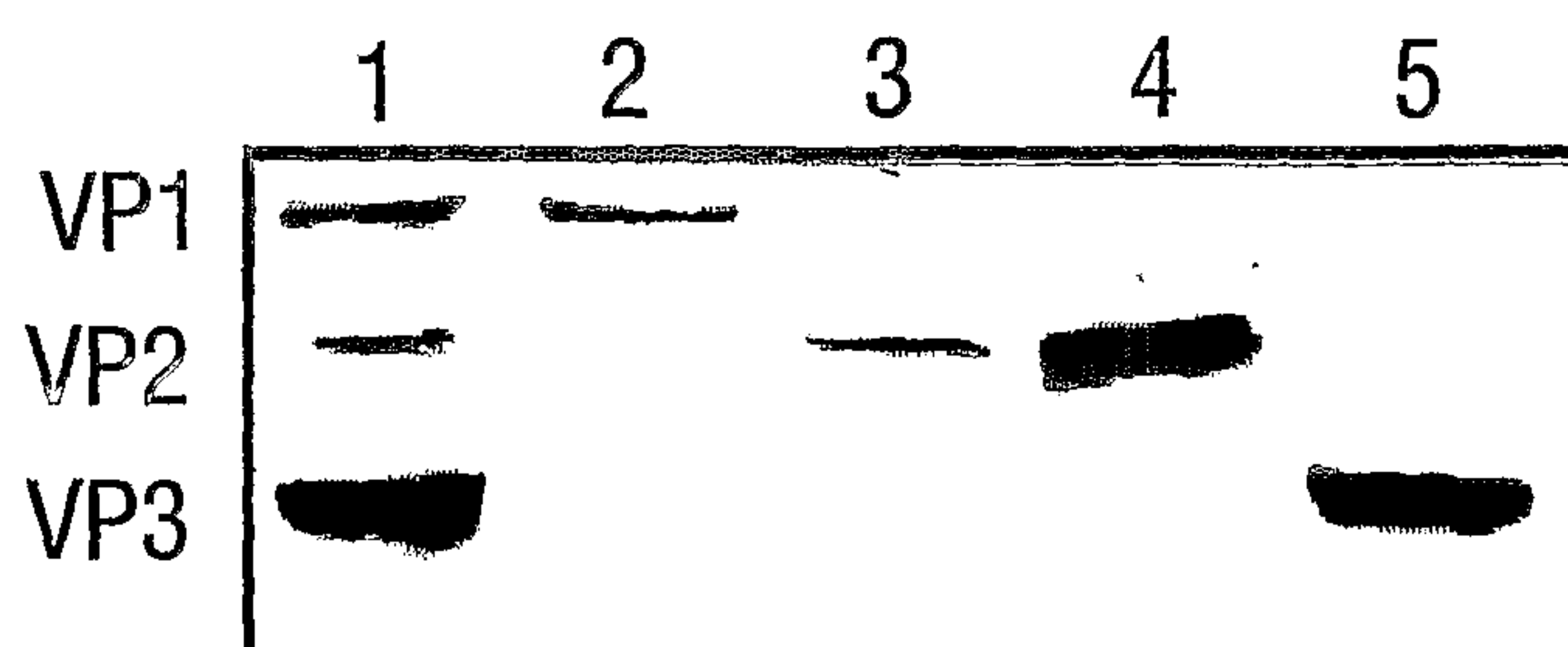
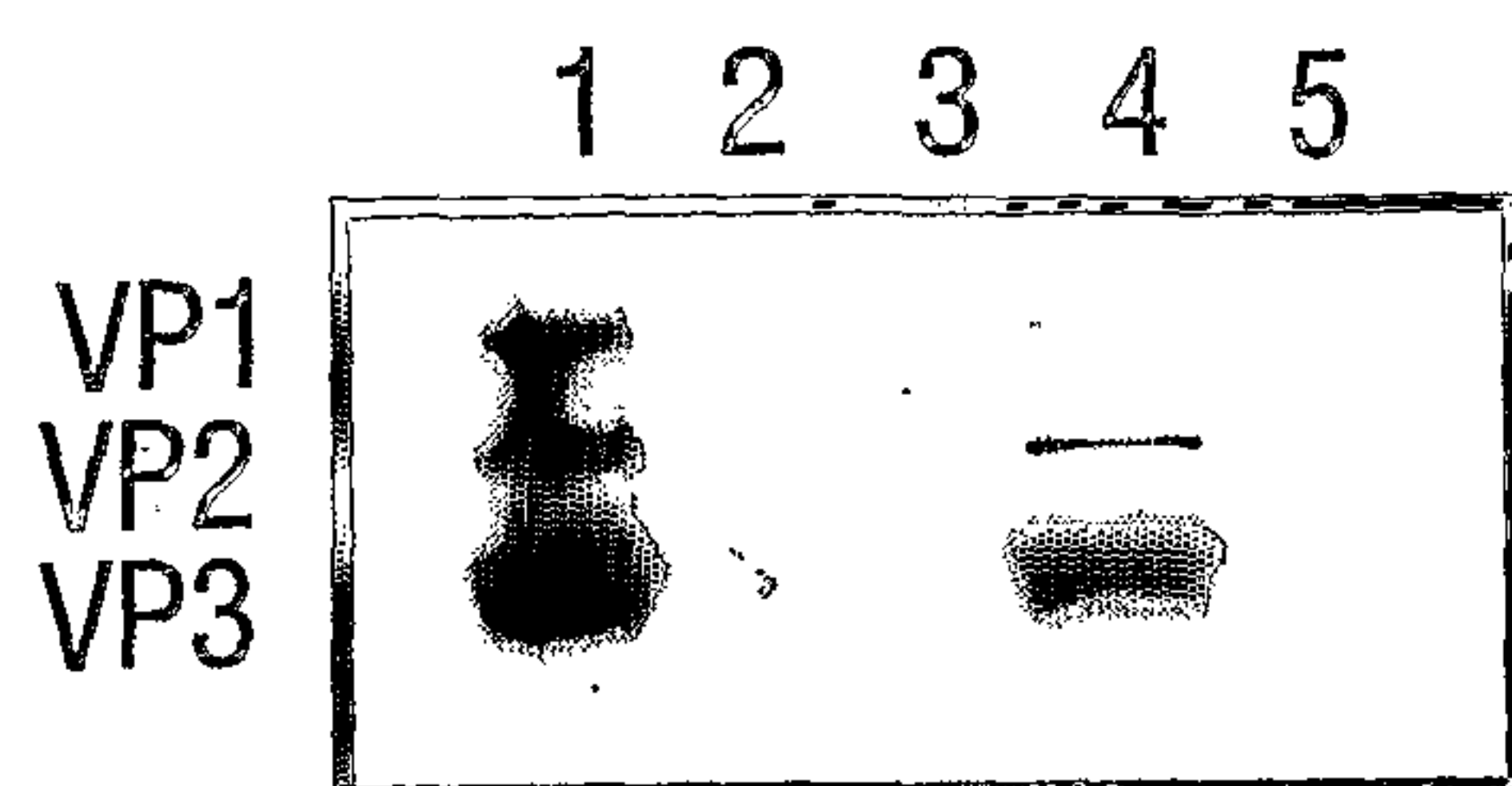
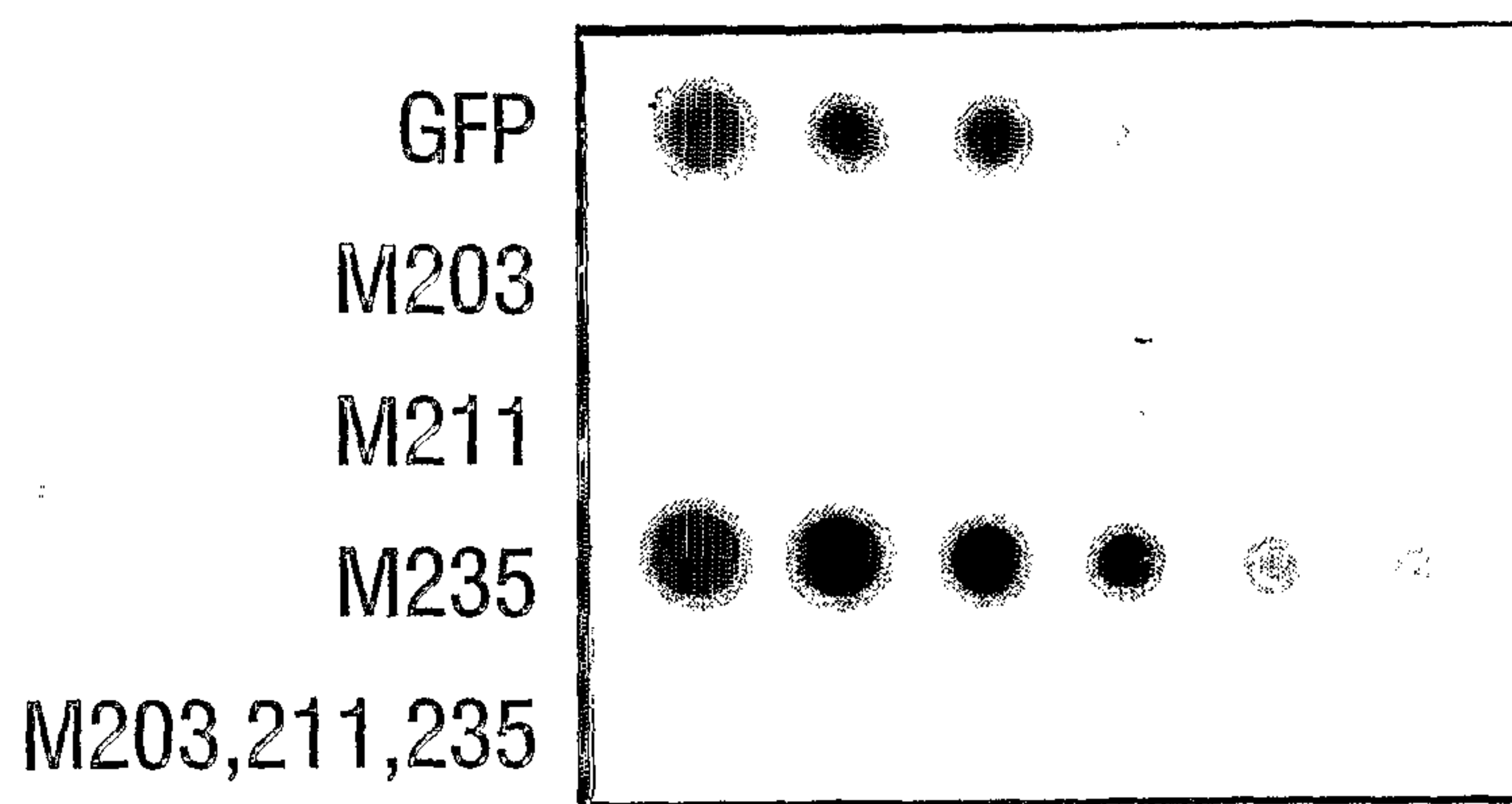
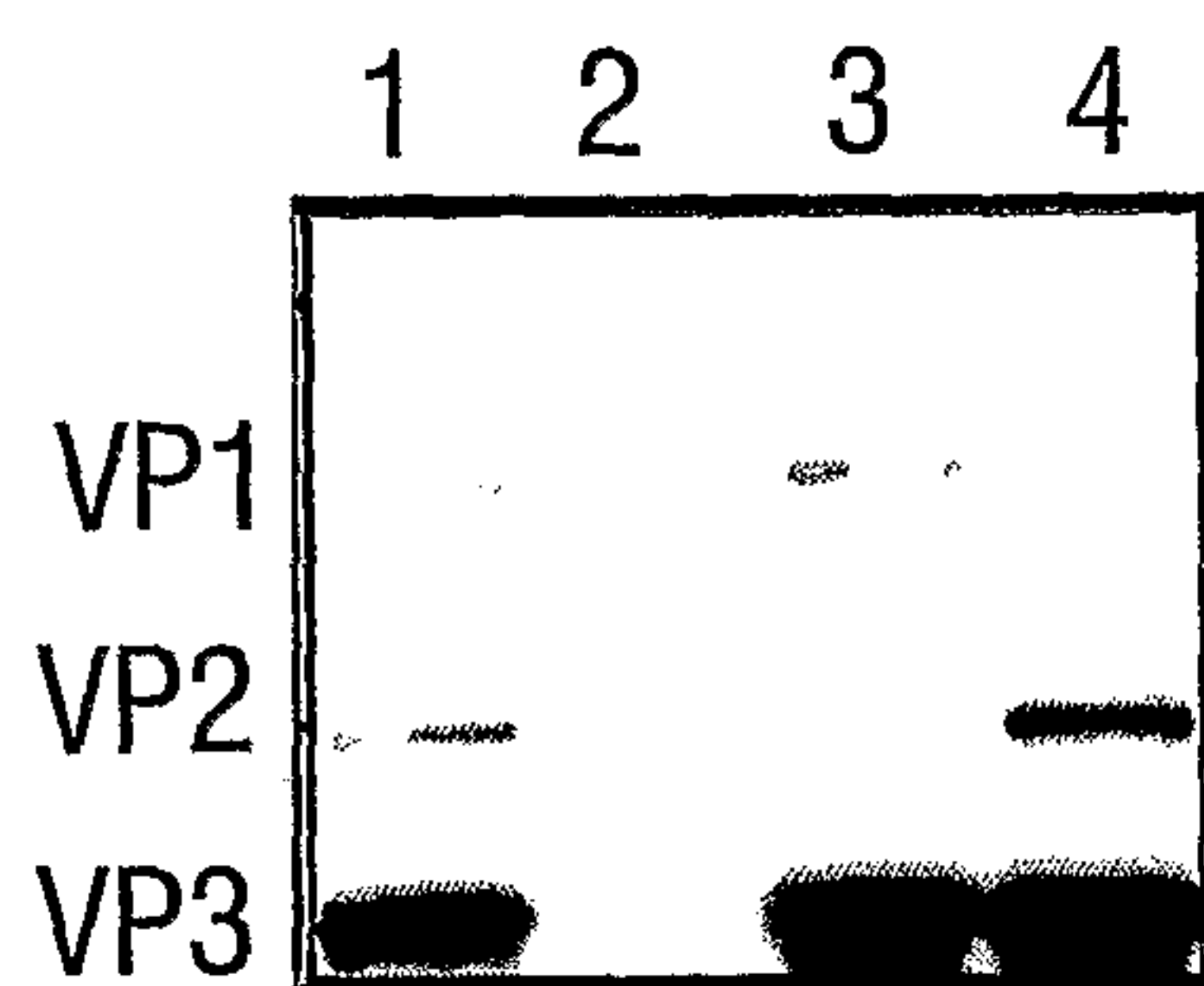
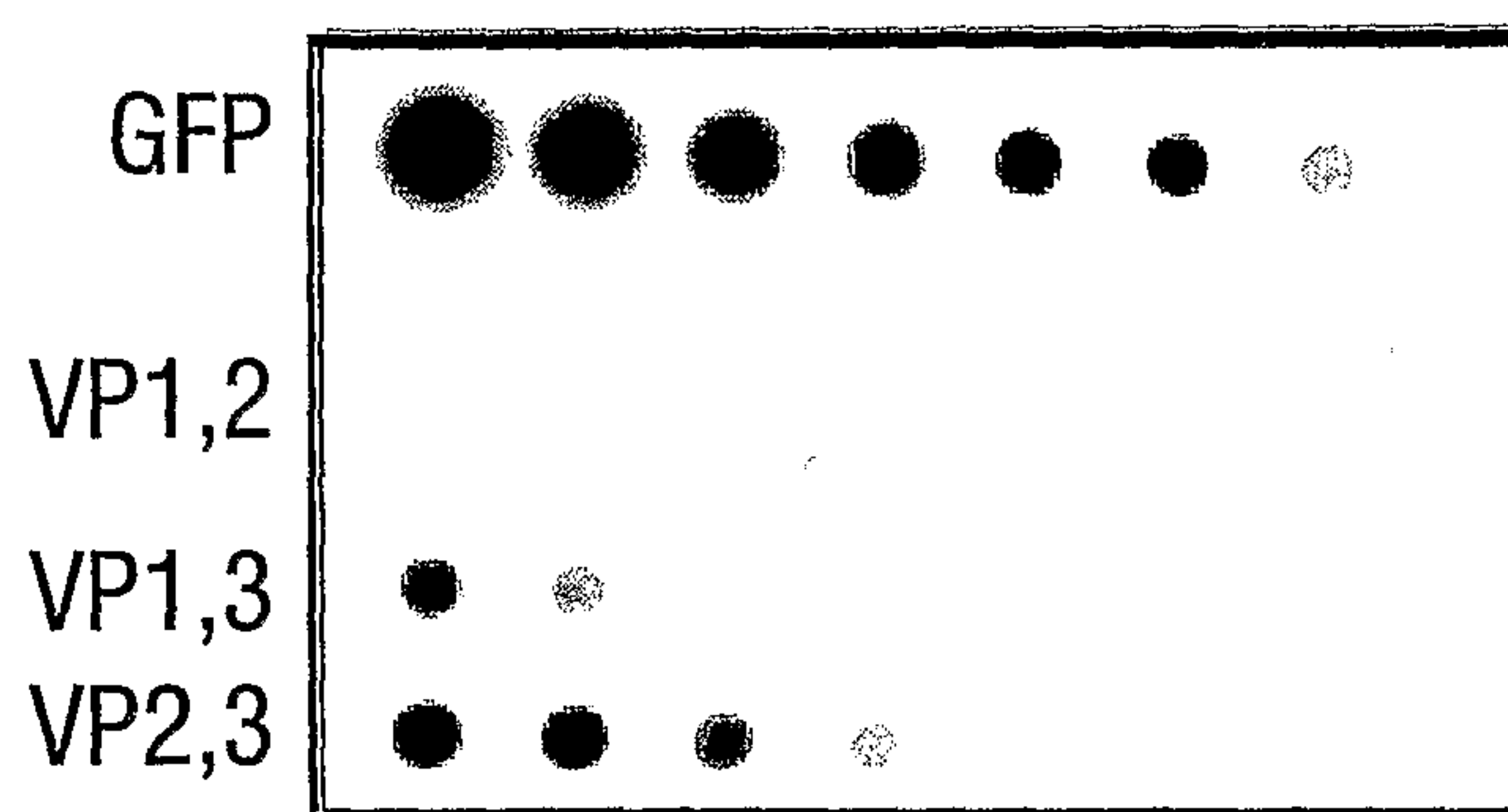
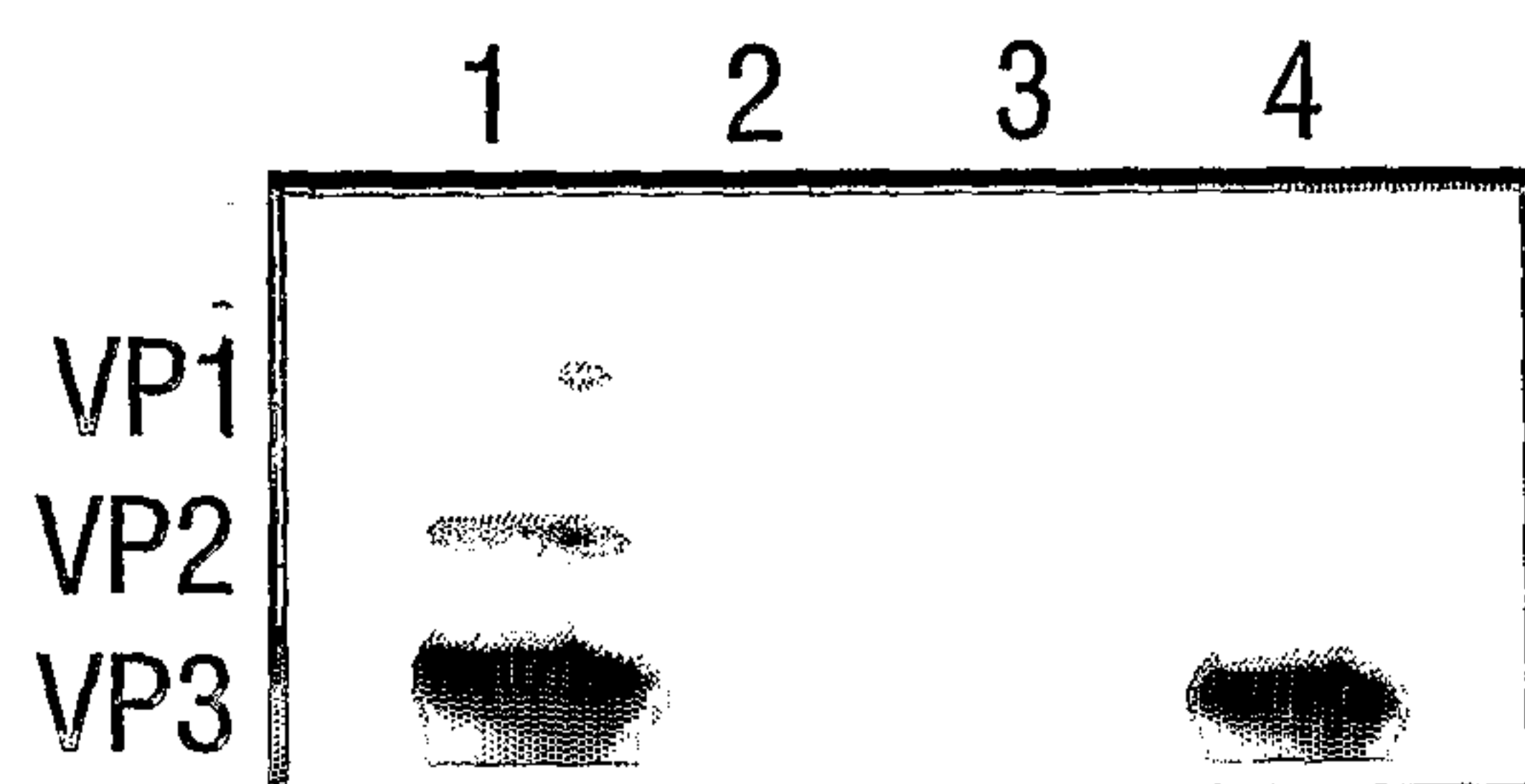
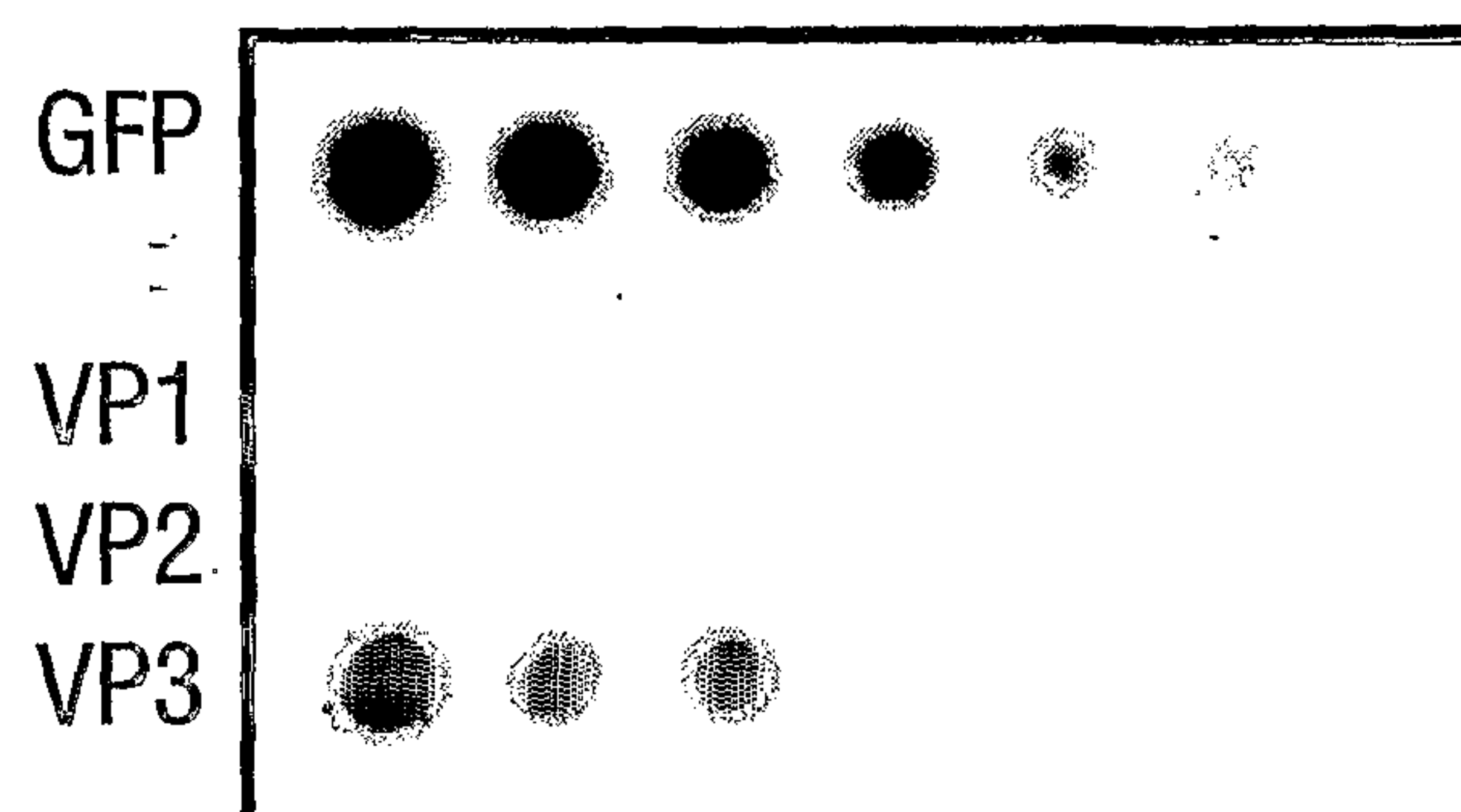


FIG. 2B

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**FIG. 3A-1****FIG. 3A-2****FIG. 3B-1****FIG. 3B-2****FIG. 3C-1****FIG. 3C-2**

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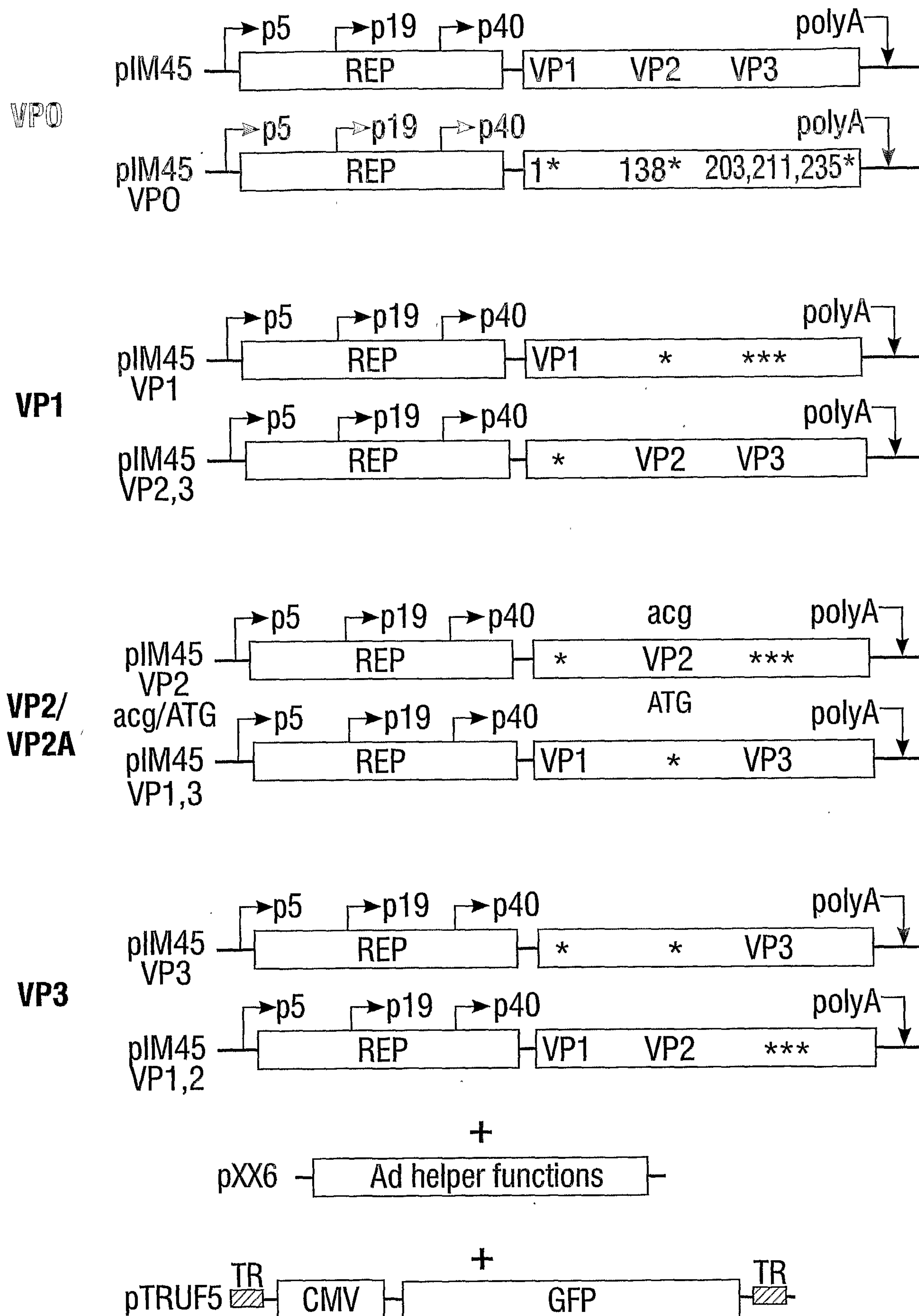


FIG. 4

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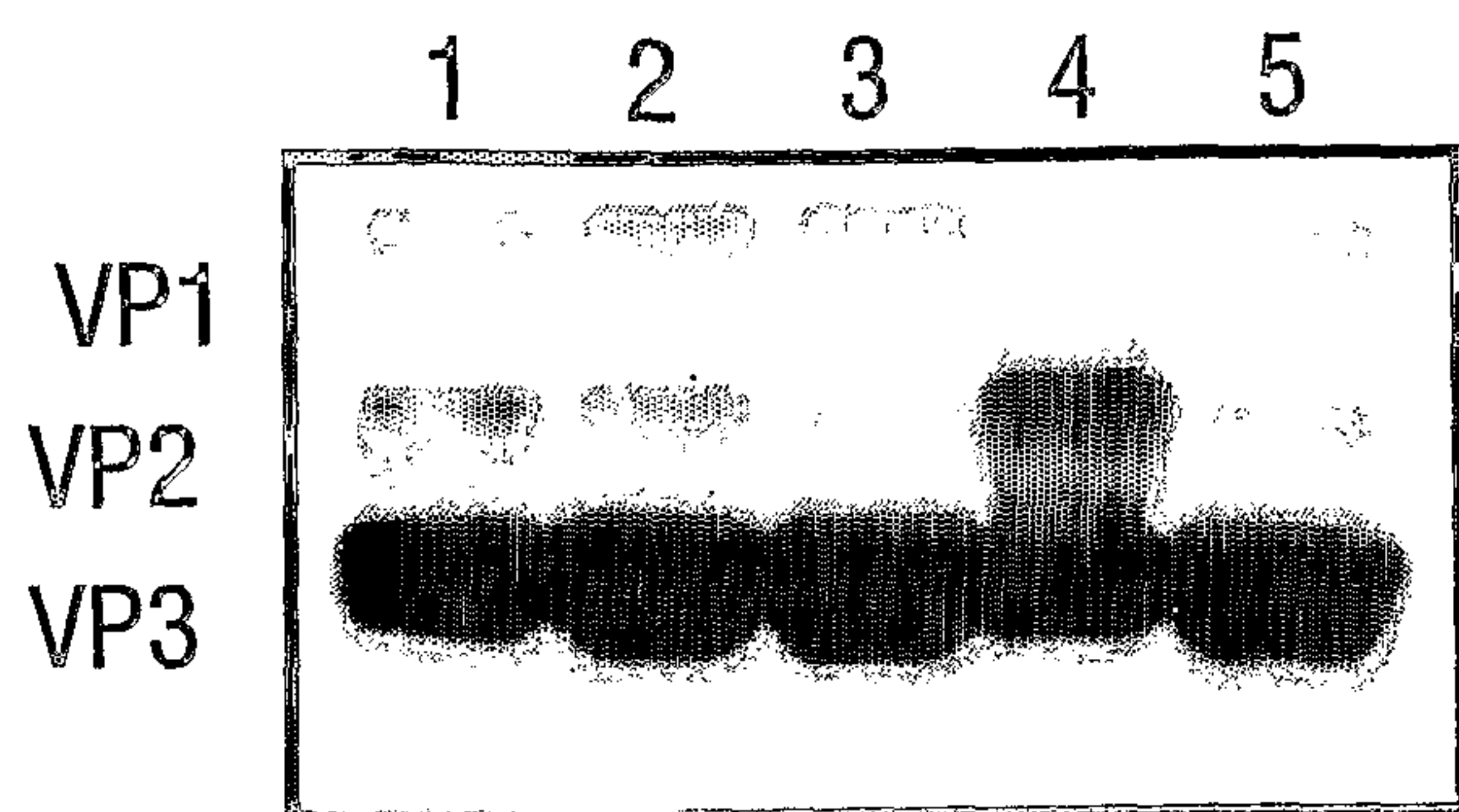


FIG. 5A-1

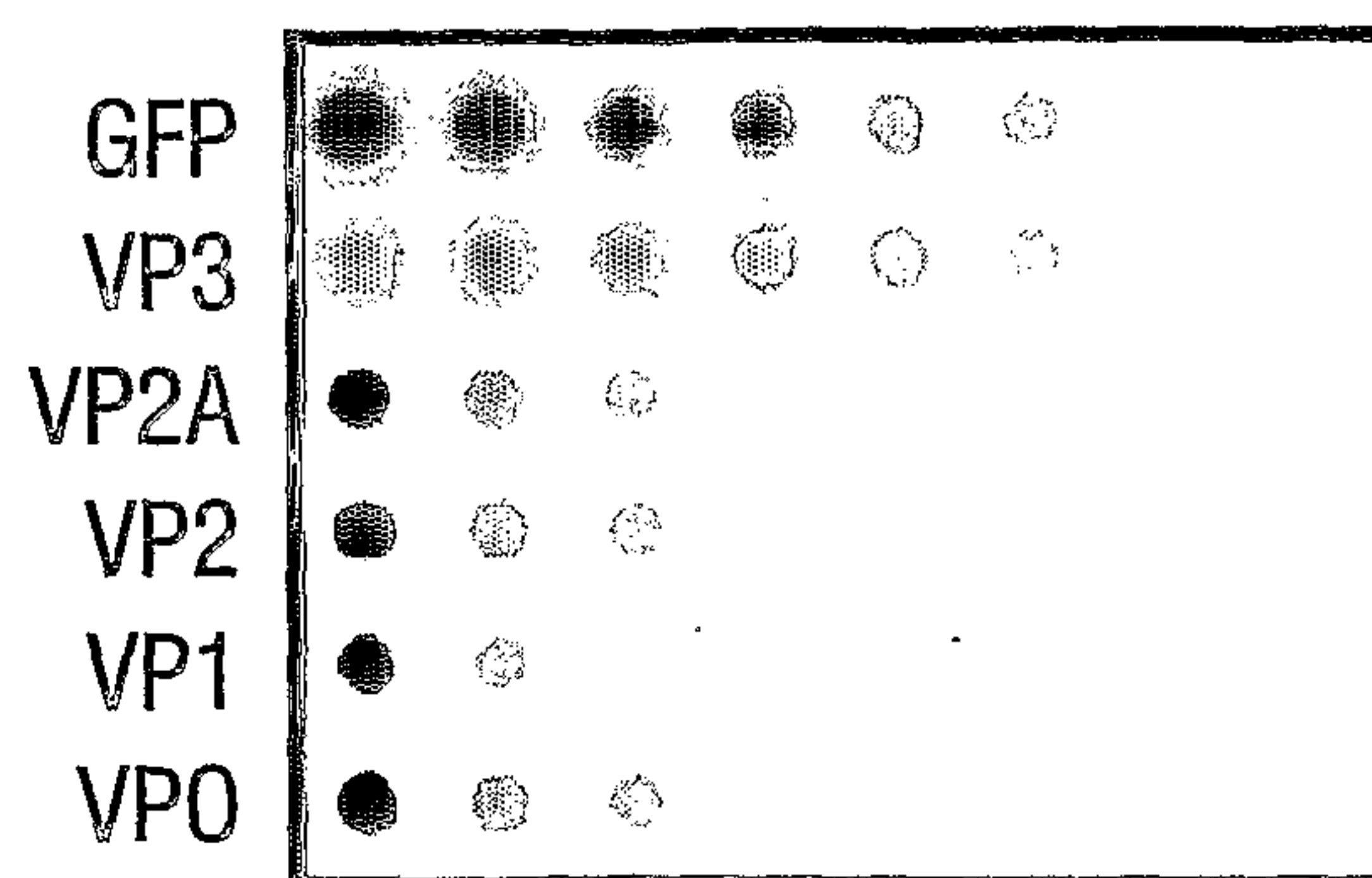


FIG. 5A-2

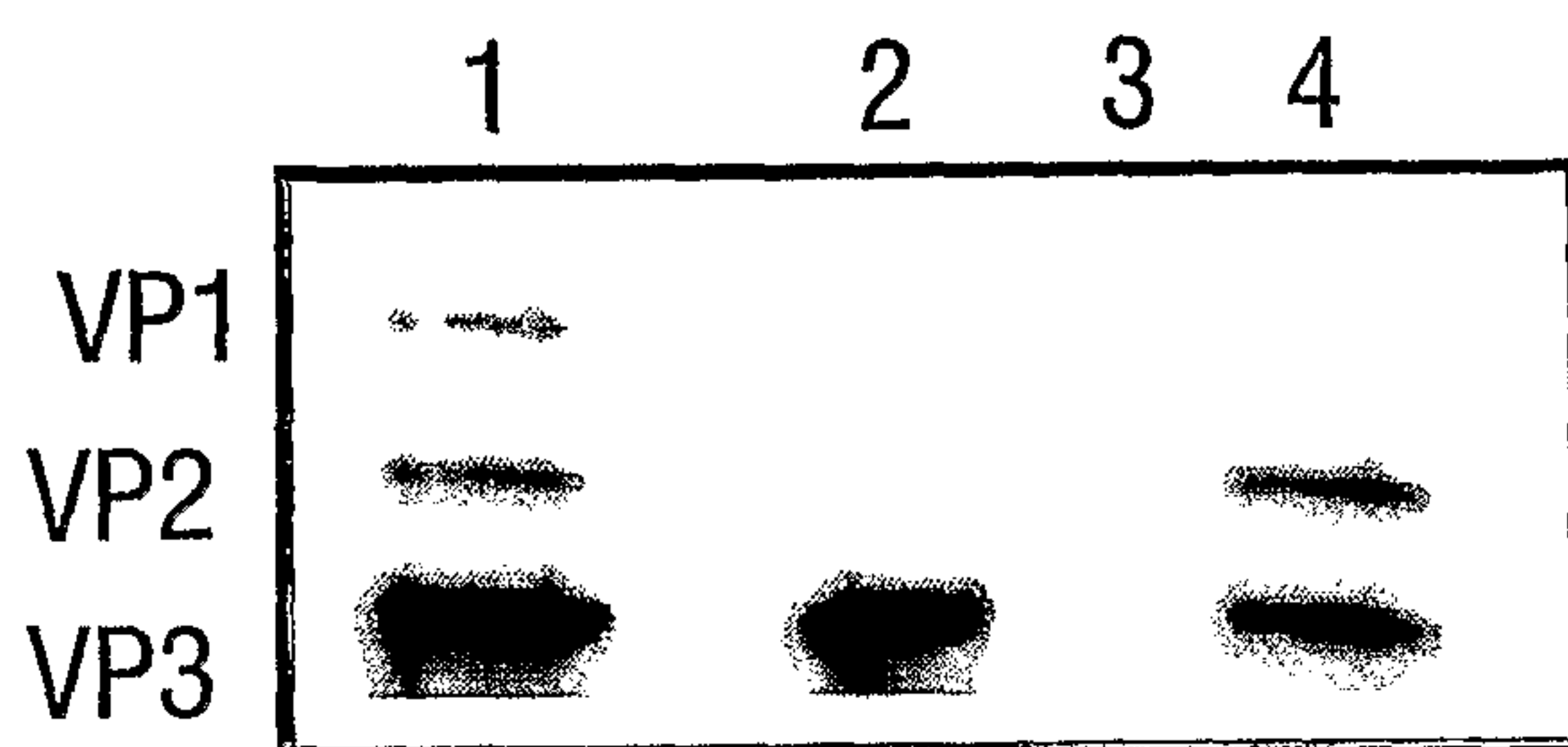


FIG. 5B-1

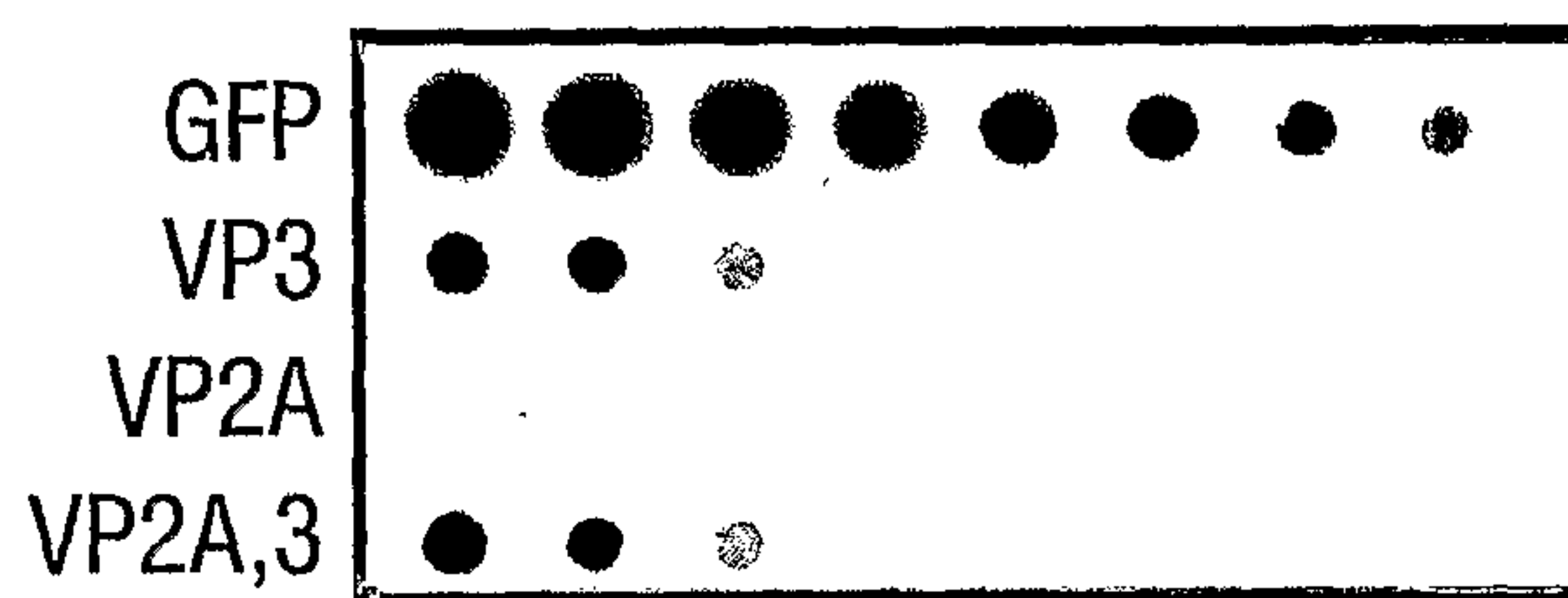


FIG. 5B-2

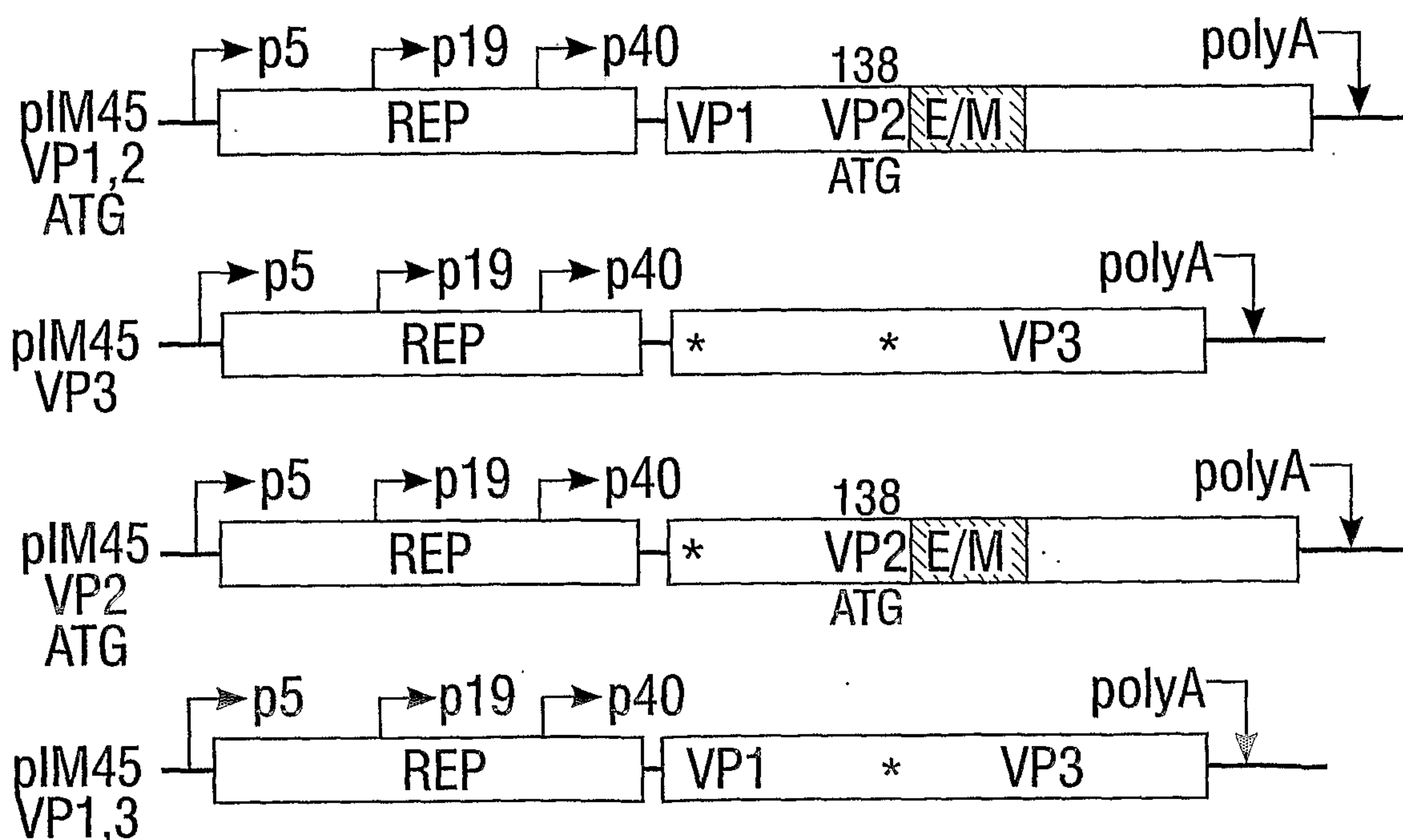
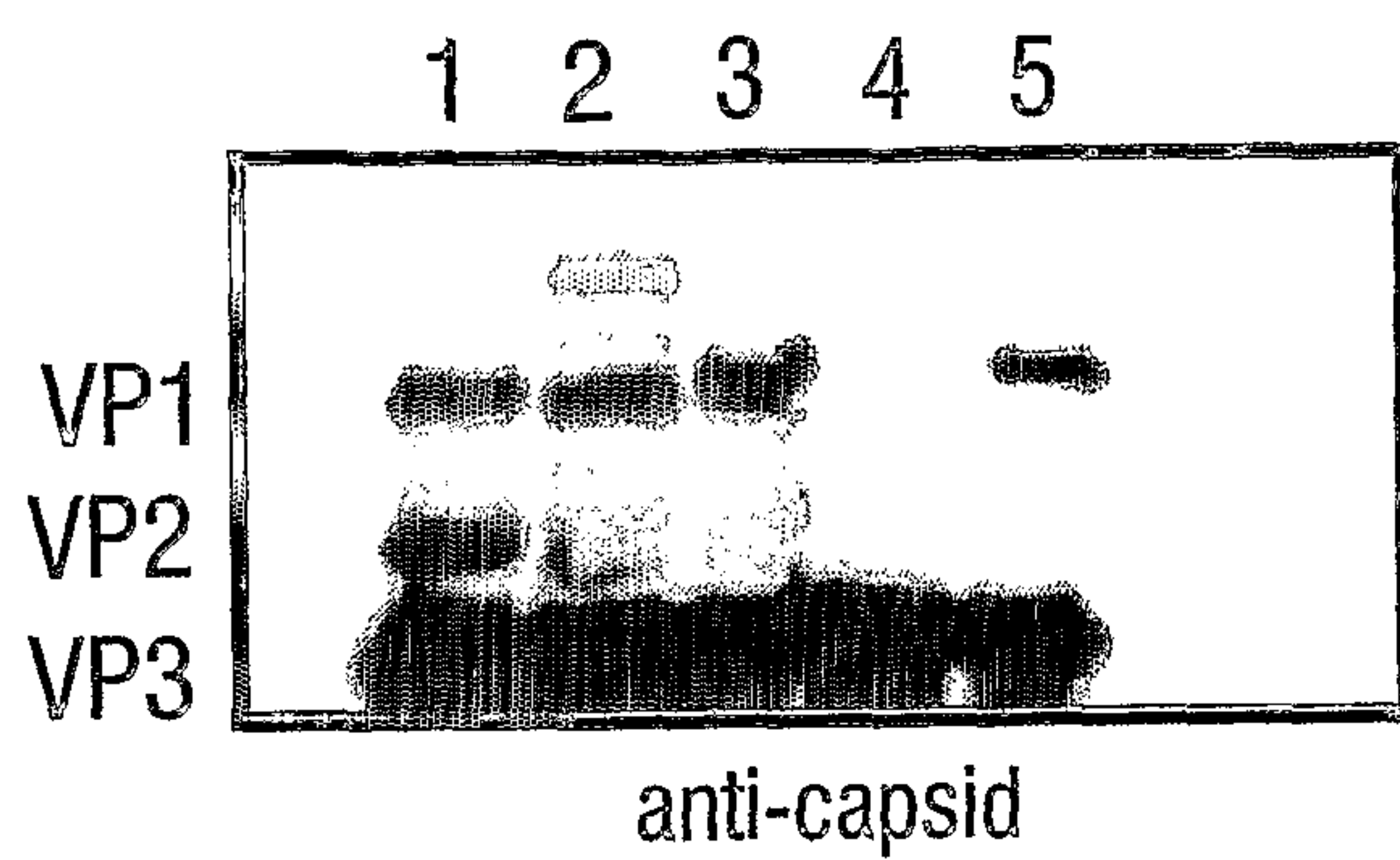
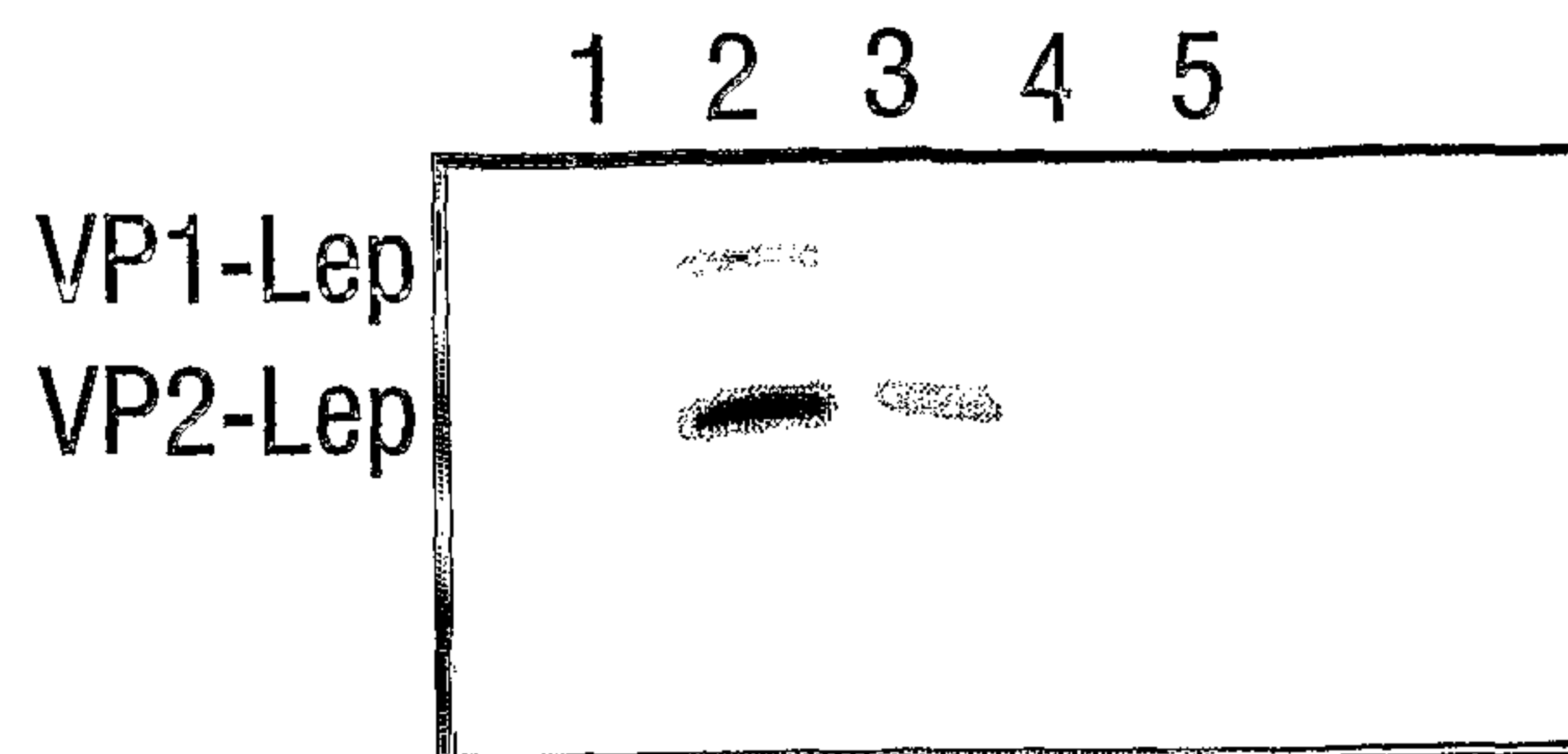


FIG. 6A

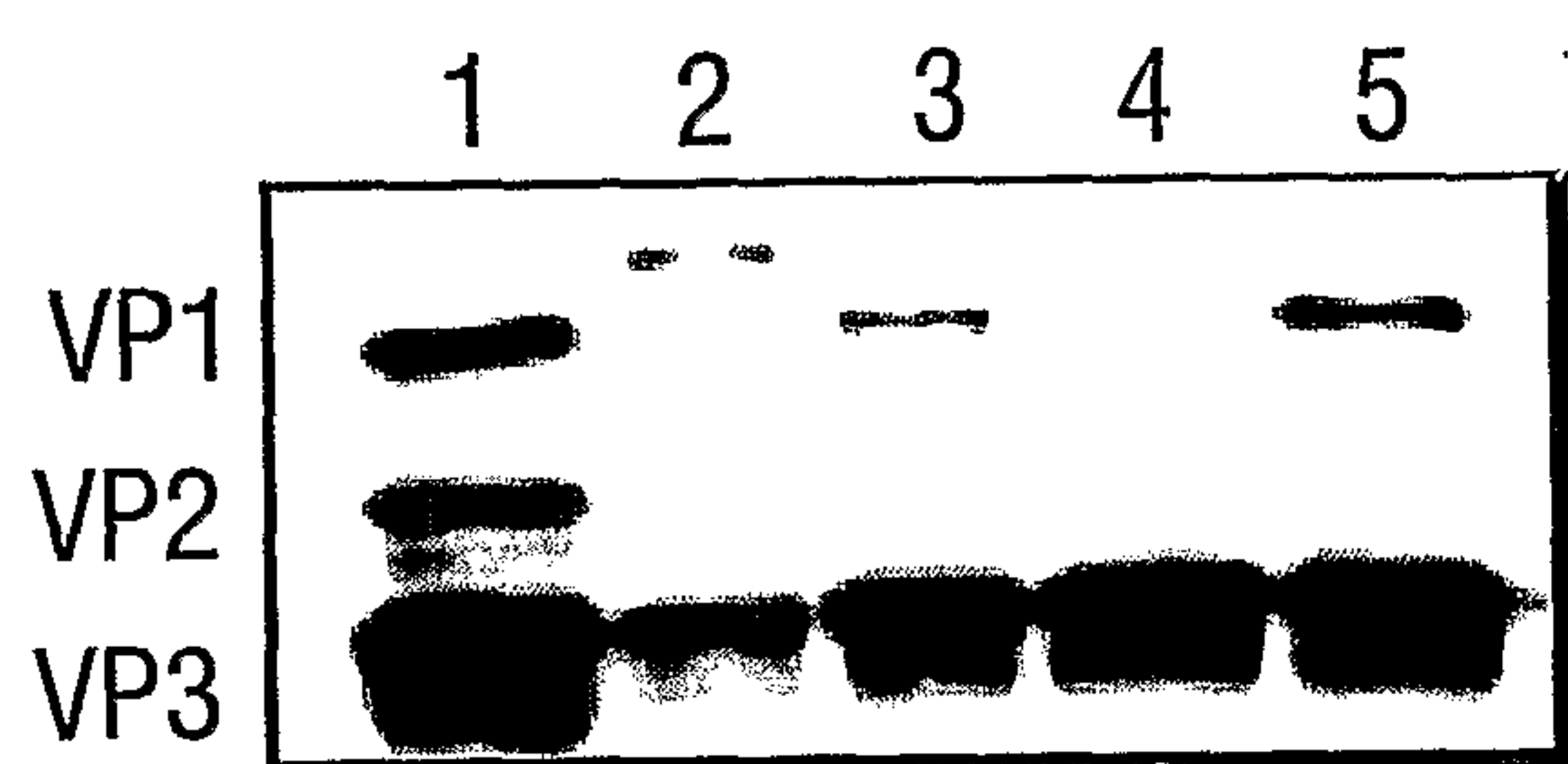
6/22



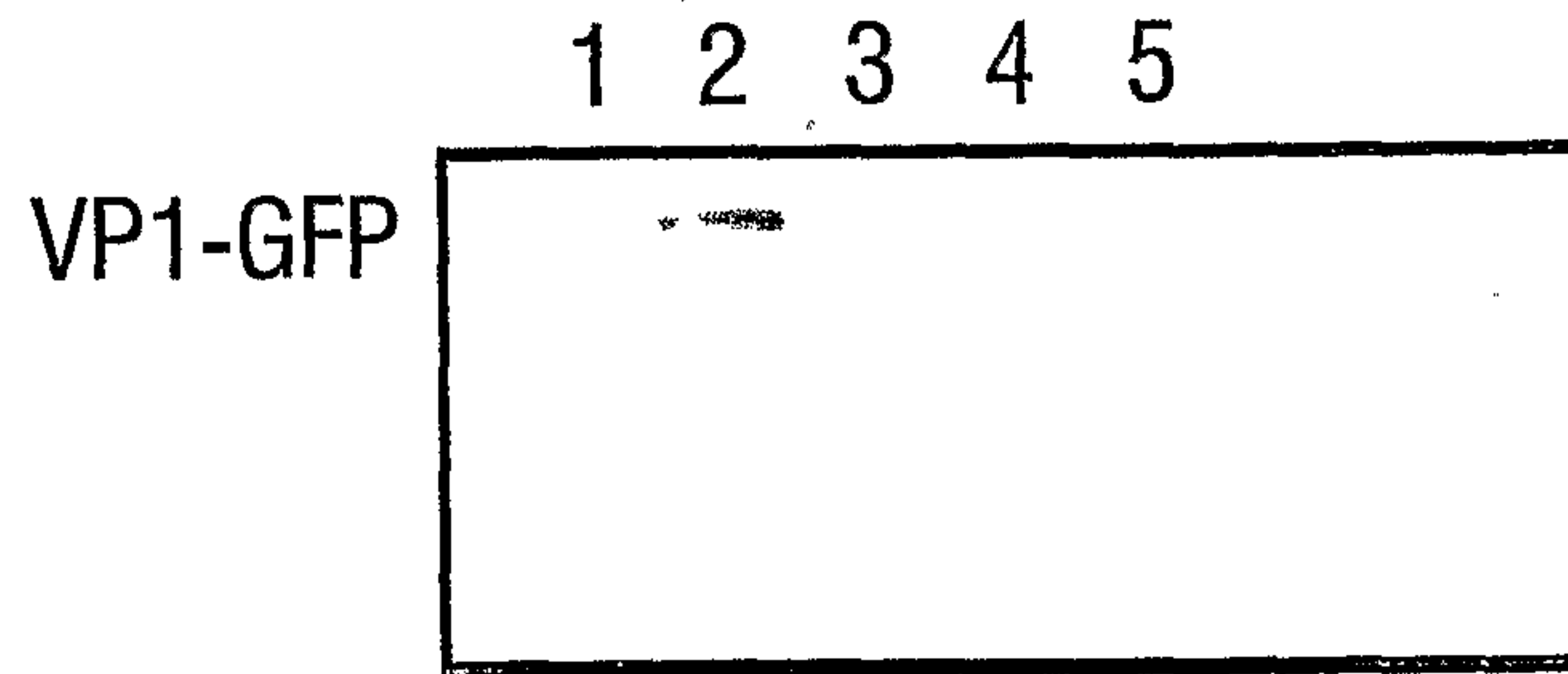
anti-capsid

FIG. 6B-1

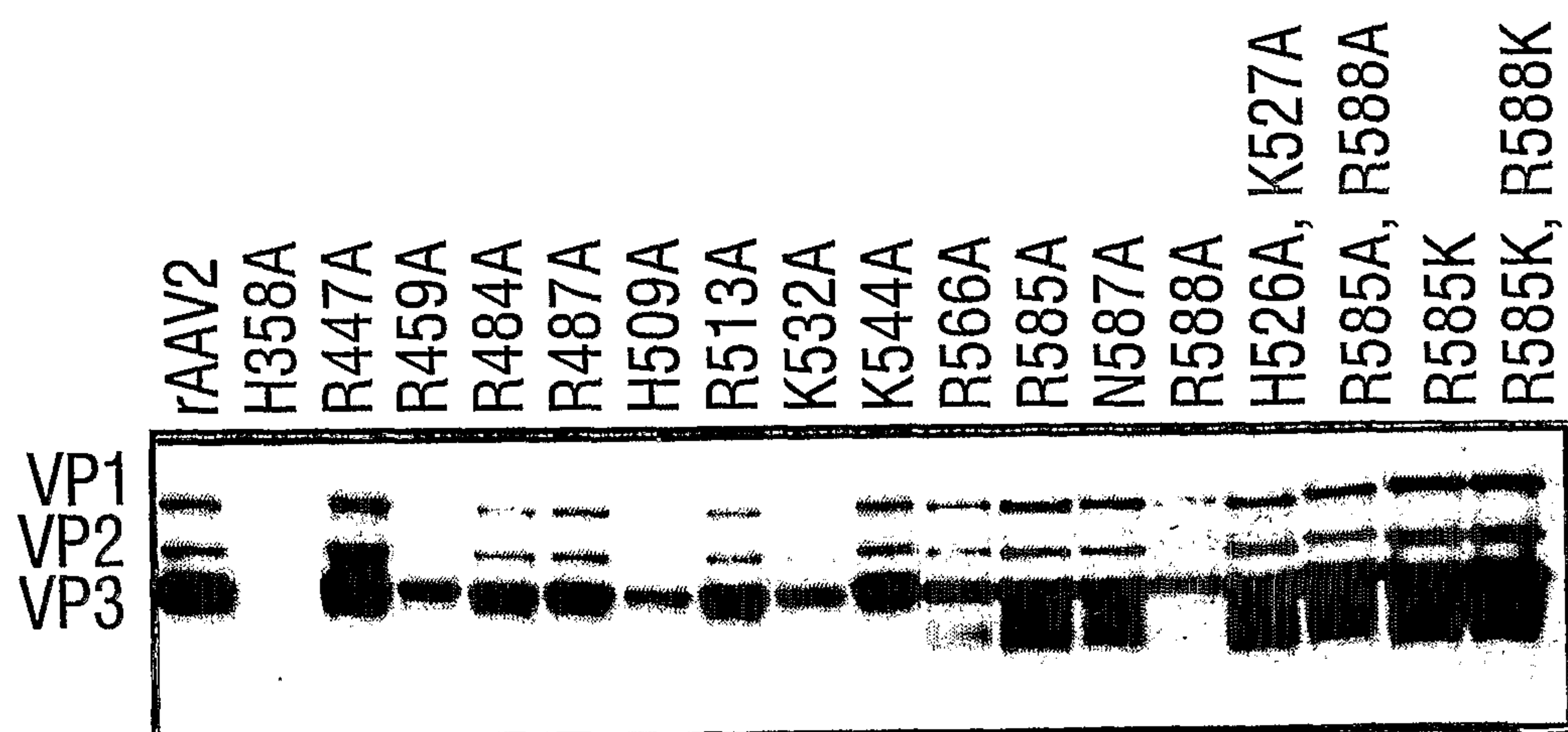
anti-Leptin

FIG. 6B-2

anti-capsid

FIG. 6C-1

anti-GFP

FIG. 6C-2**FIG. 7**

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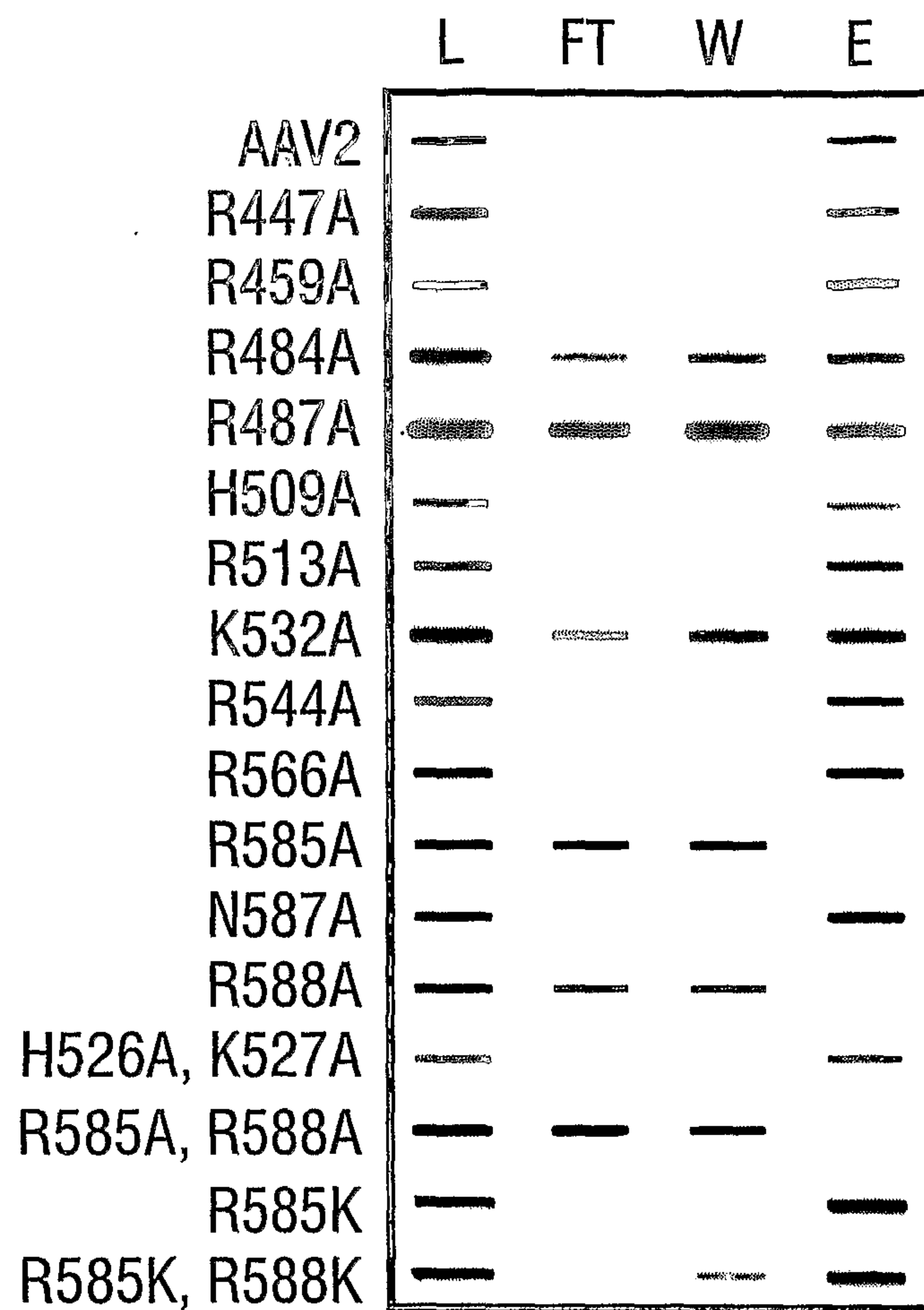


FIG. 8

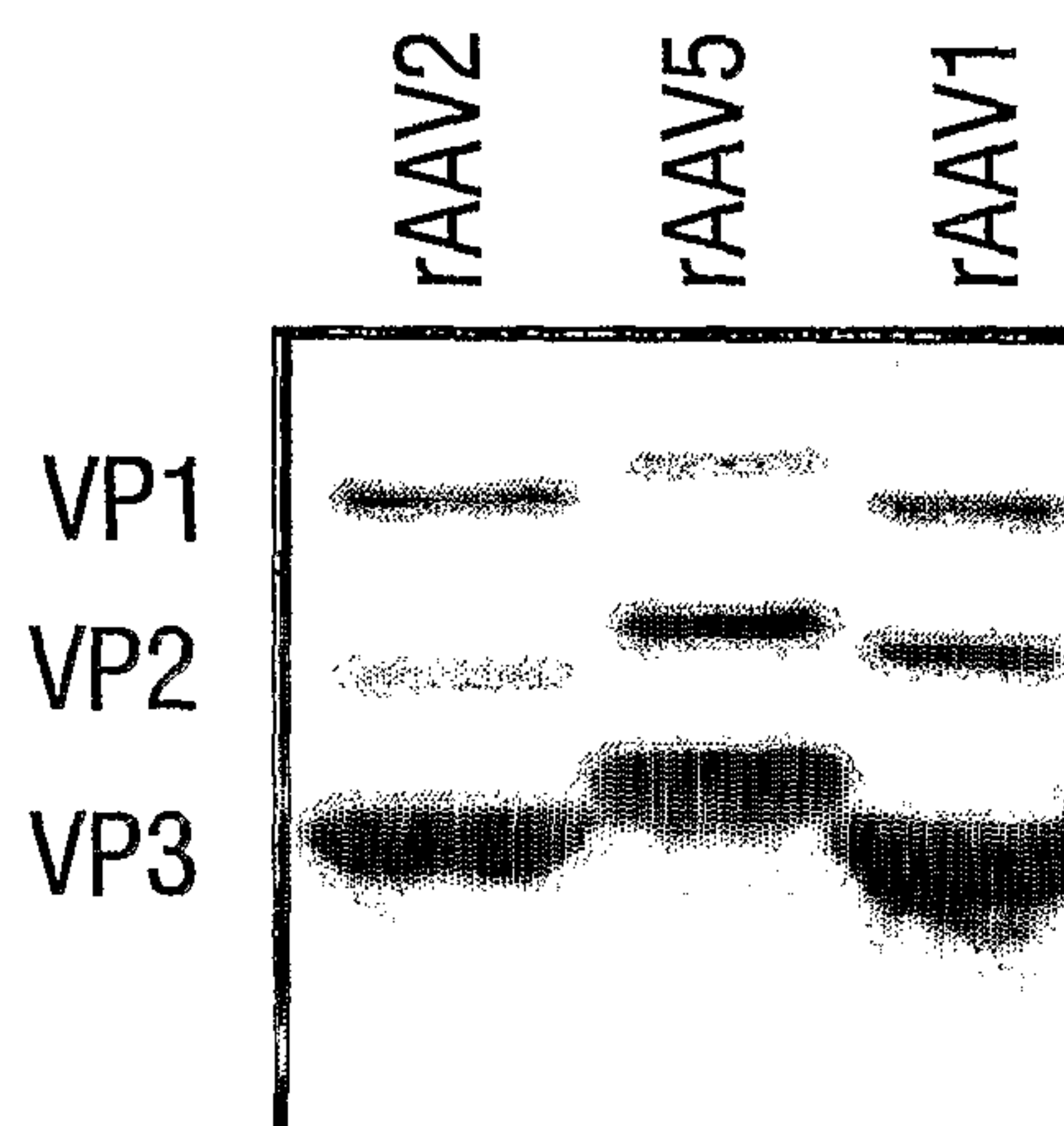


FIG. 9A

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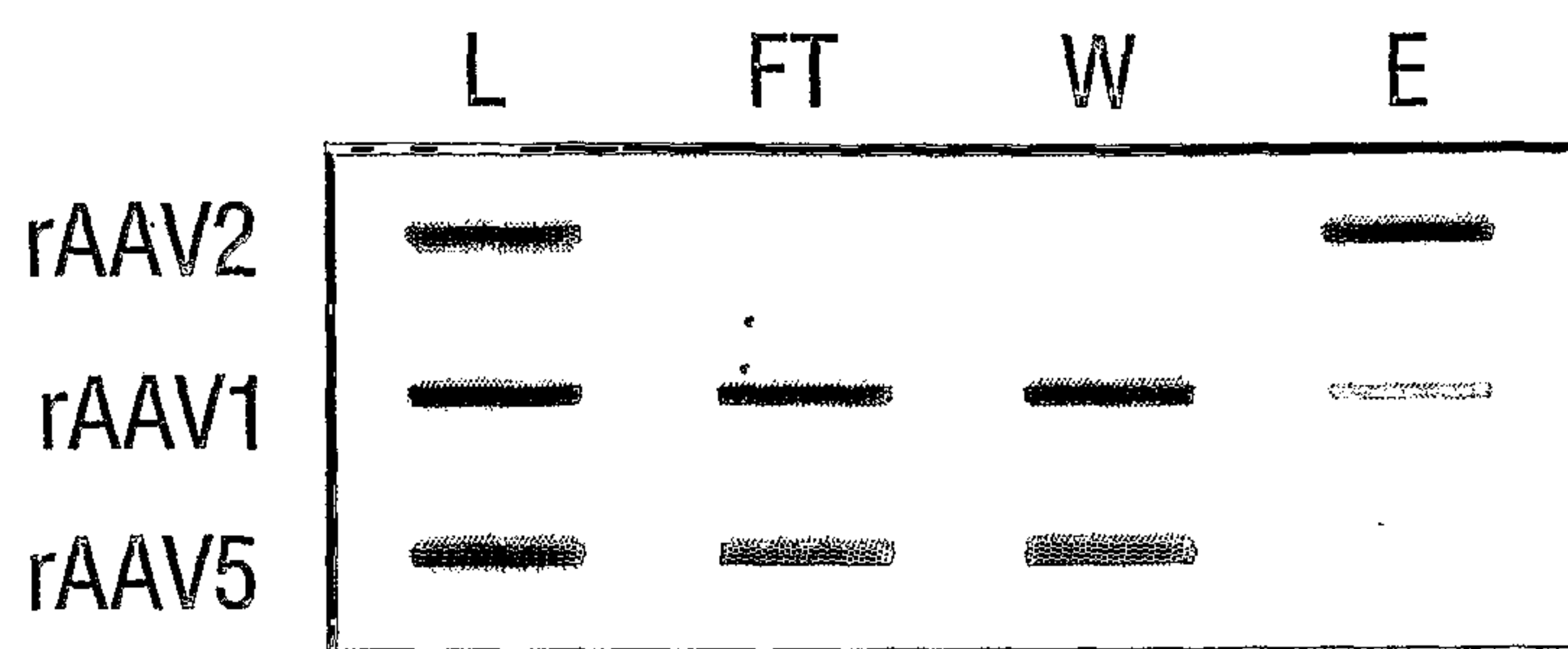


FIG. 9B

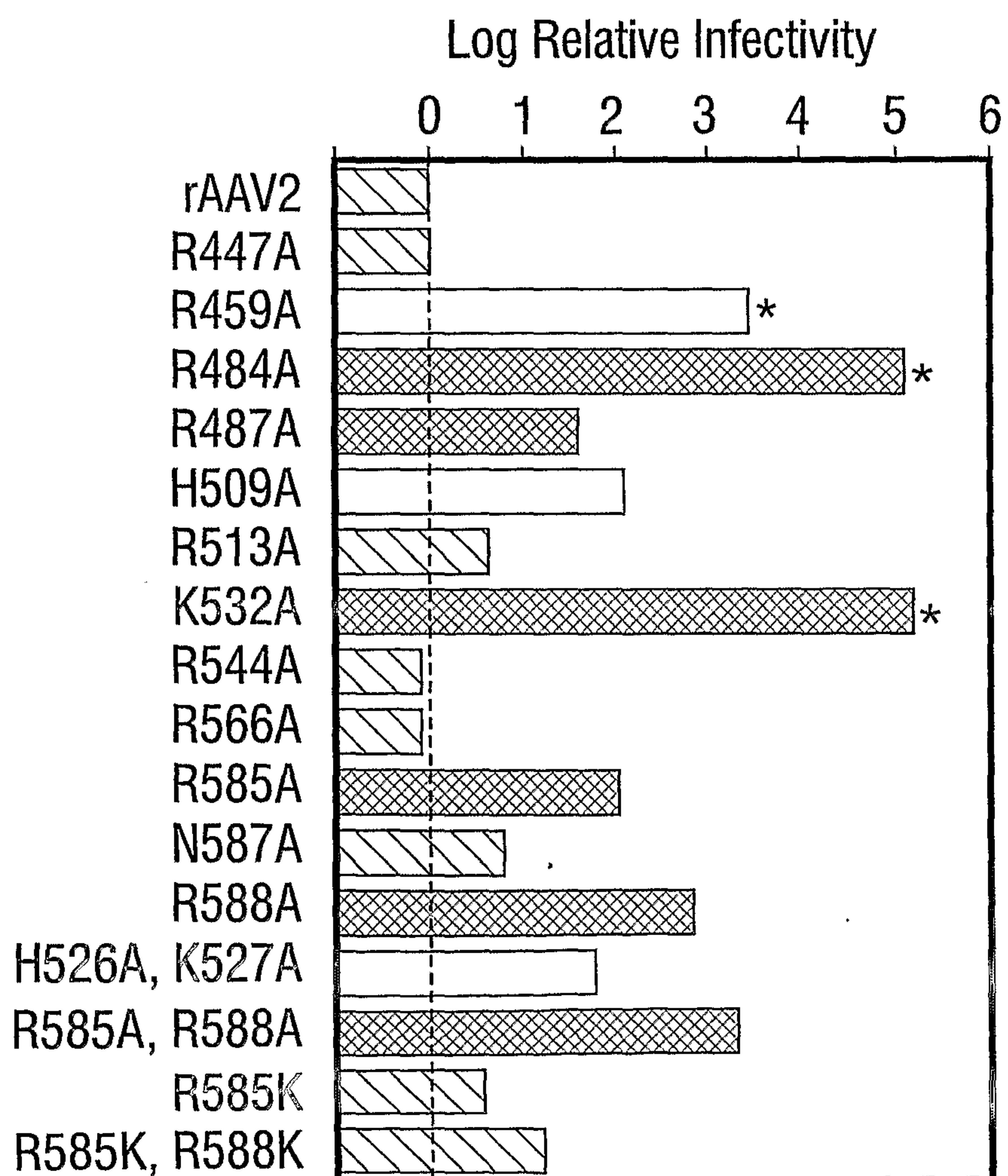


FIG. 10

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Relative Cell Number

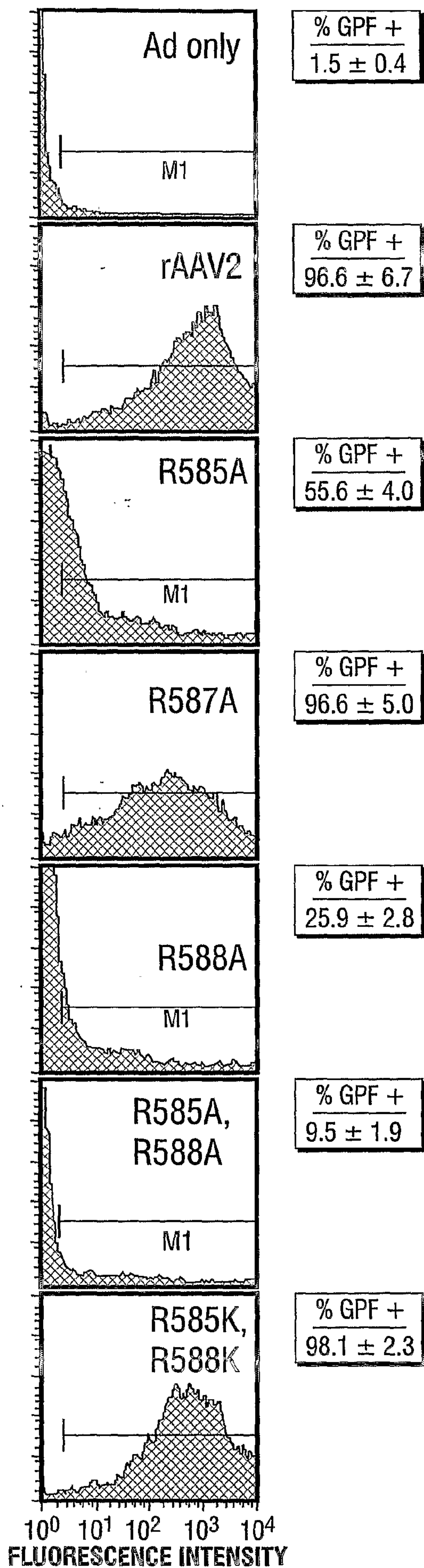
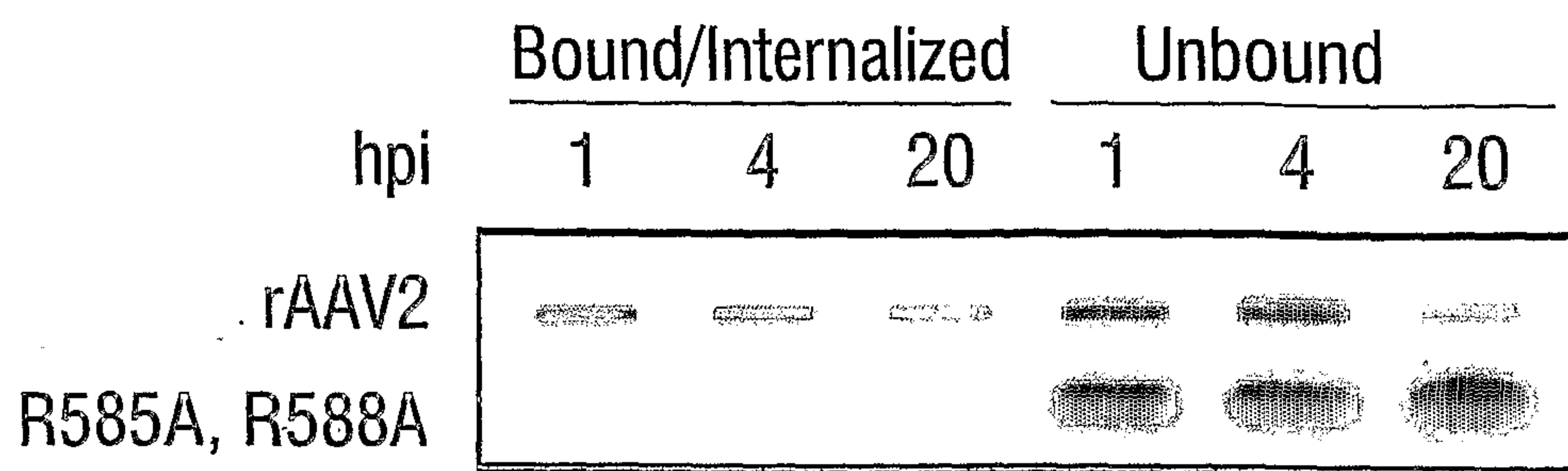
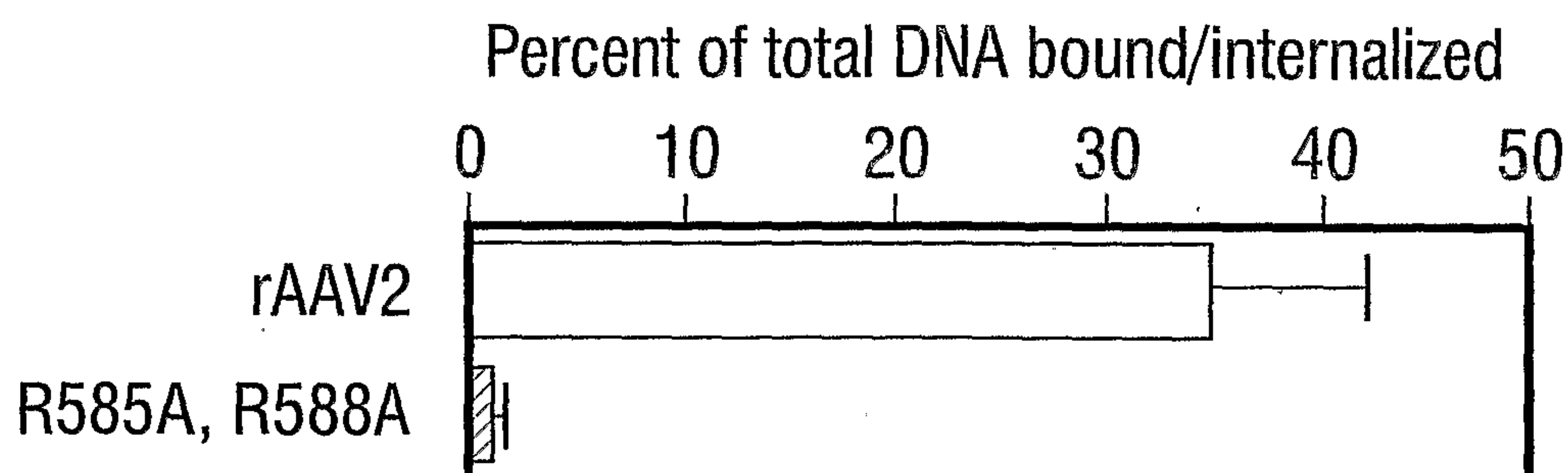
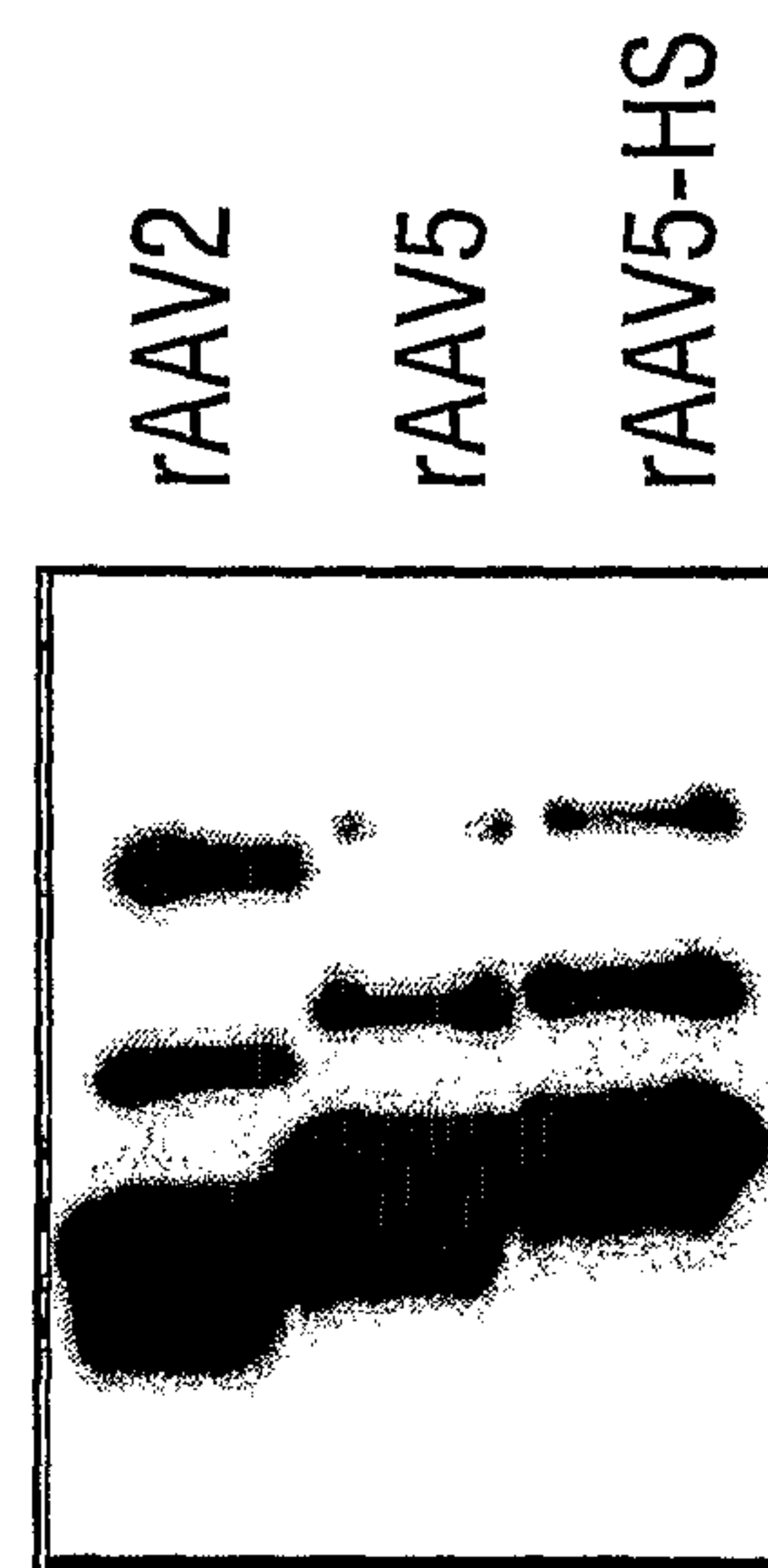
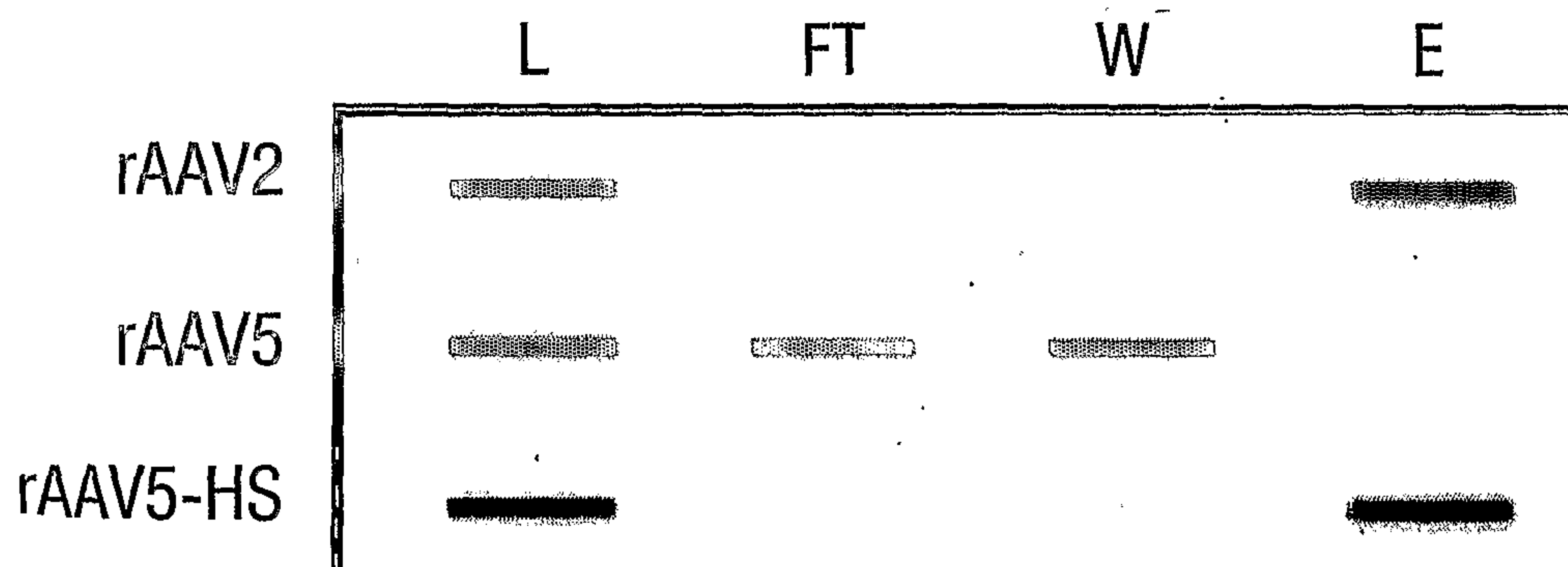


FIG. 11

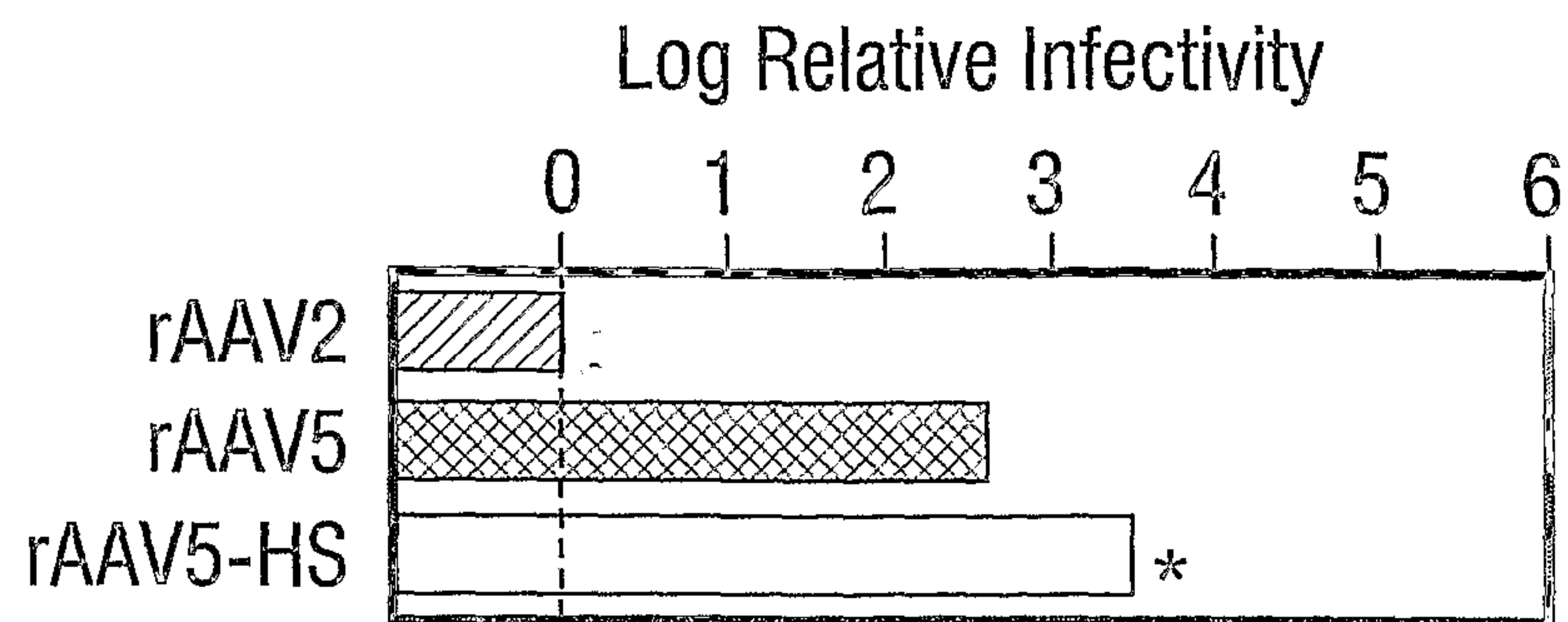
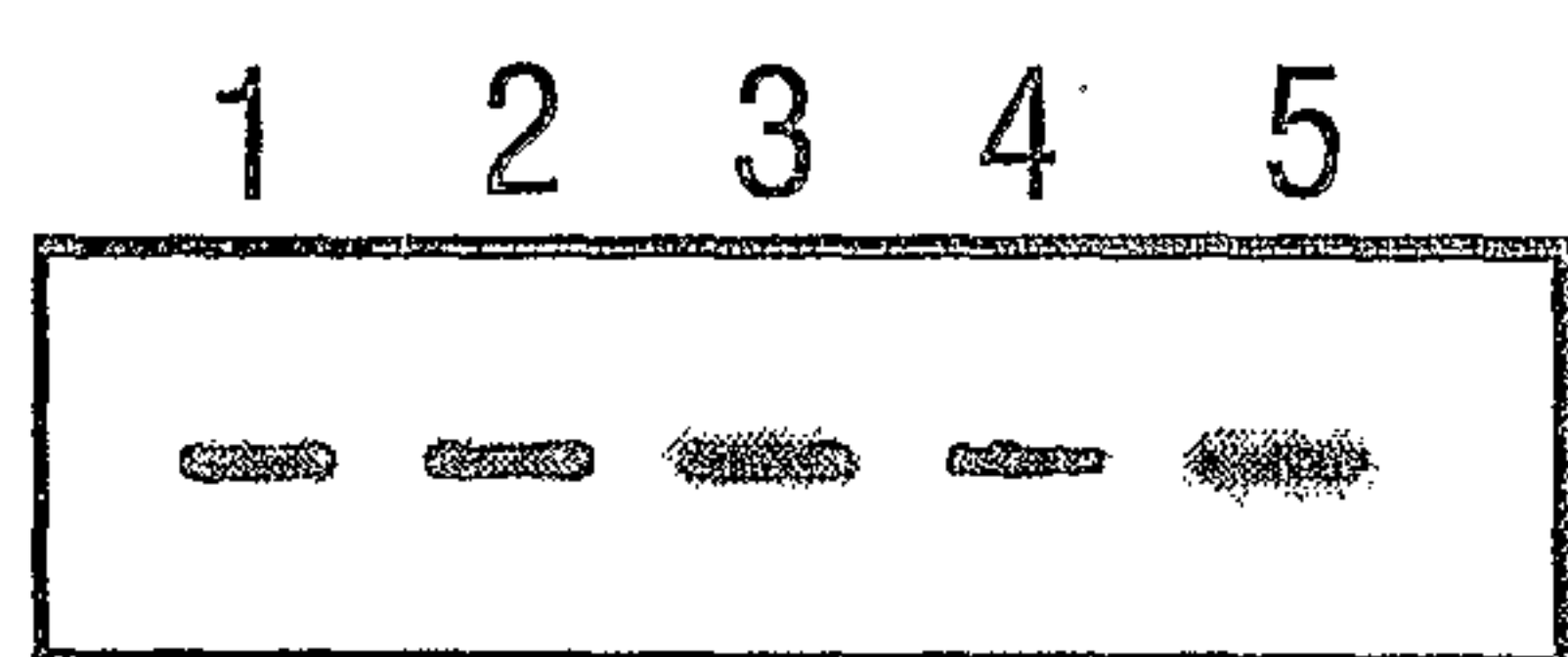
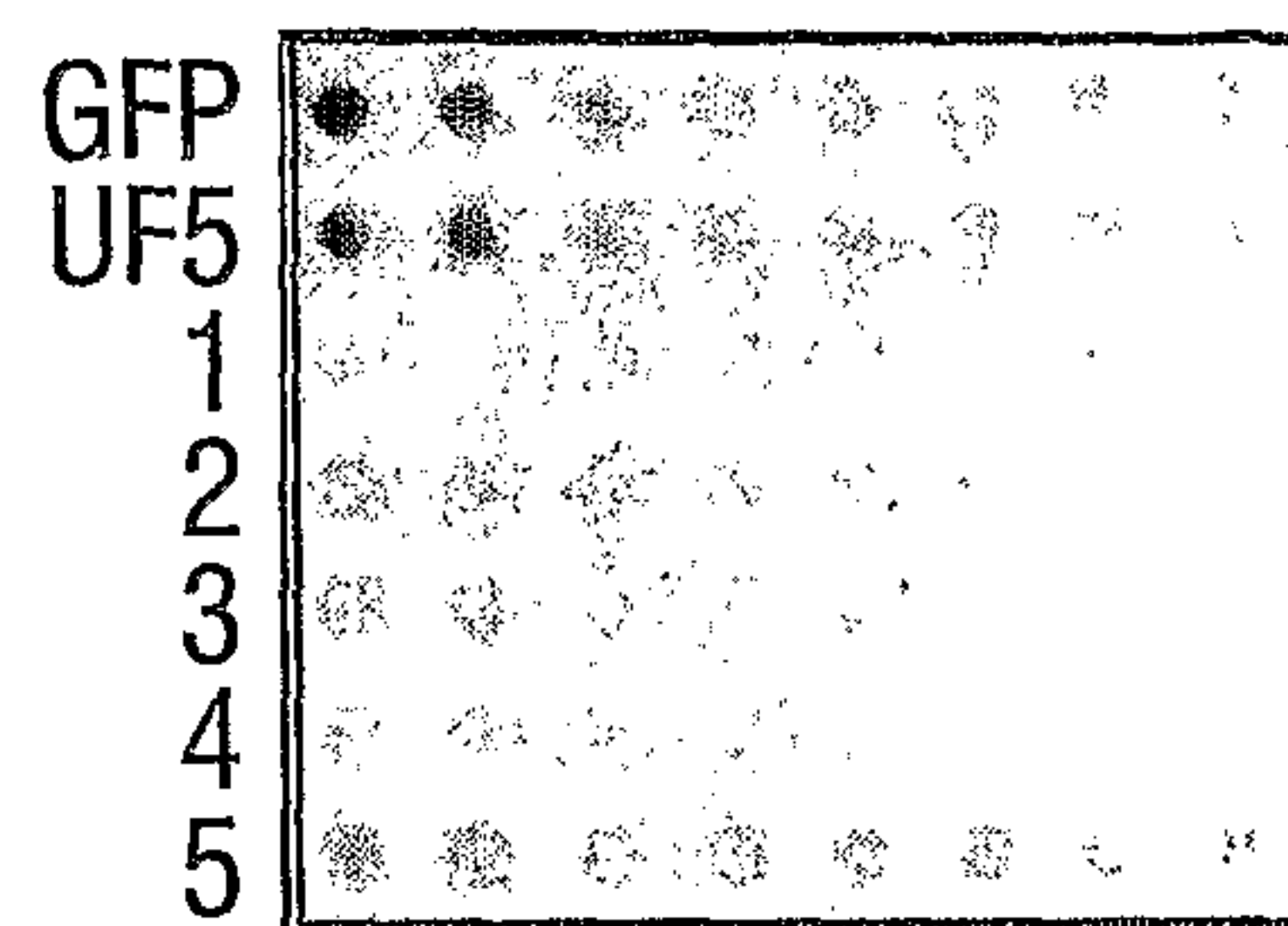
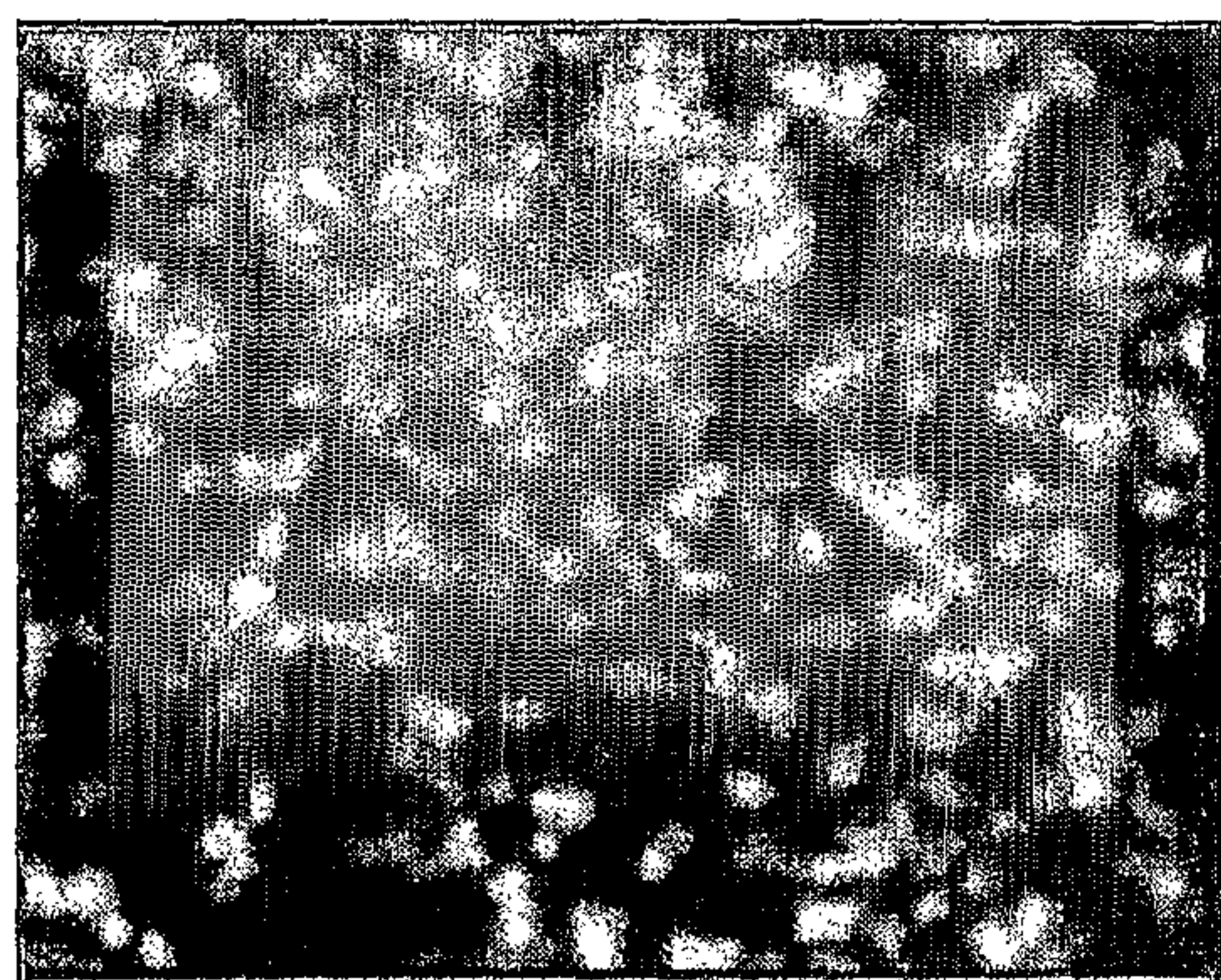
10/22**FIG. 12A****FIG. 12B**

AAV2 585 590
RGNRQA

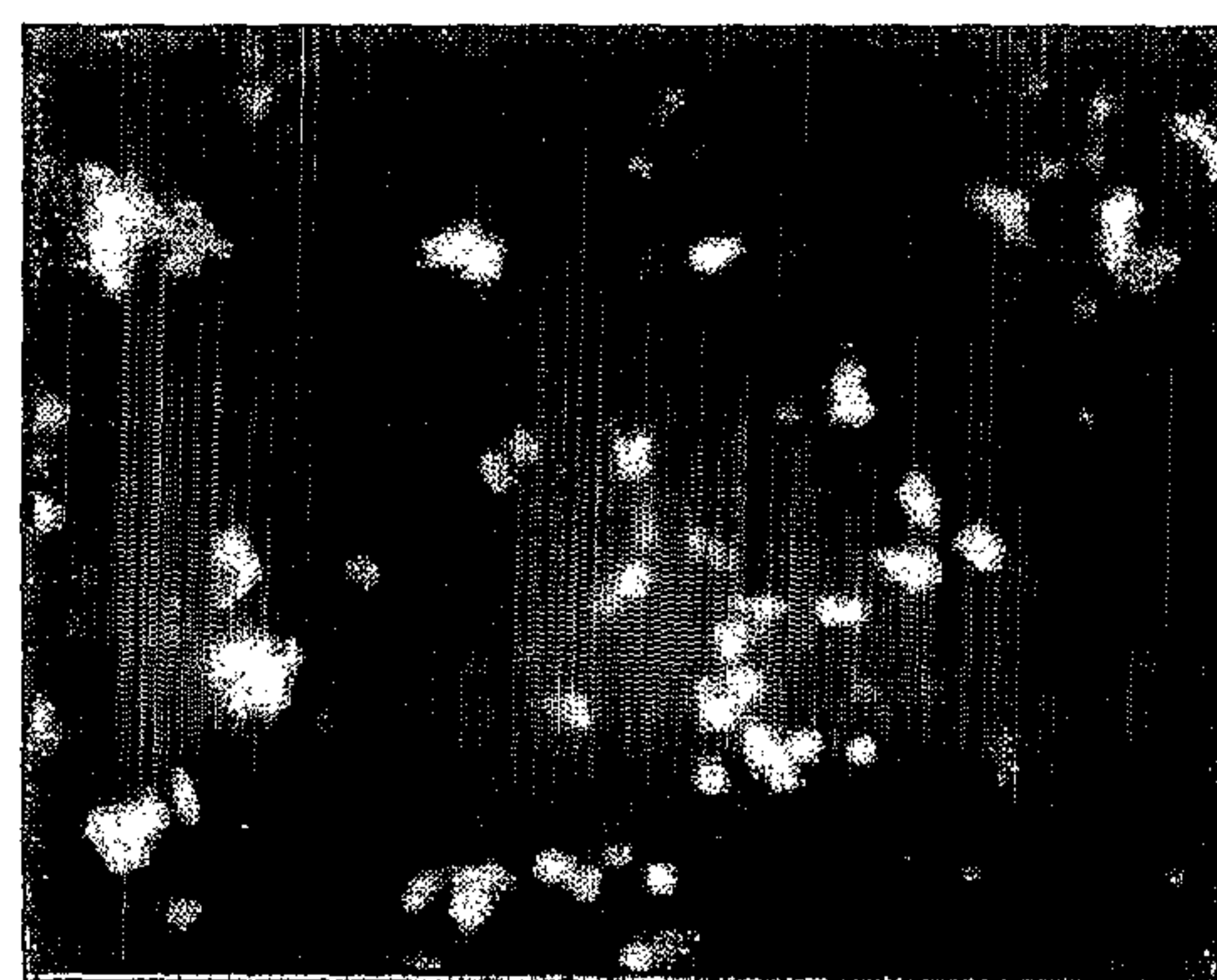
AAV5 575 580
SNSNLP

FIG. 13A**FIG. 13B****FIG. 13C**

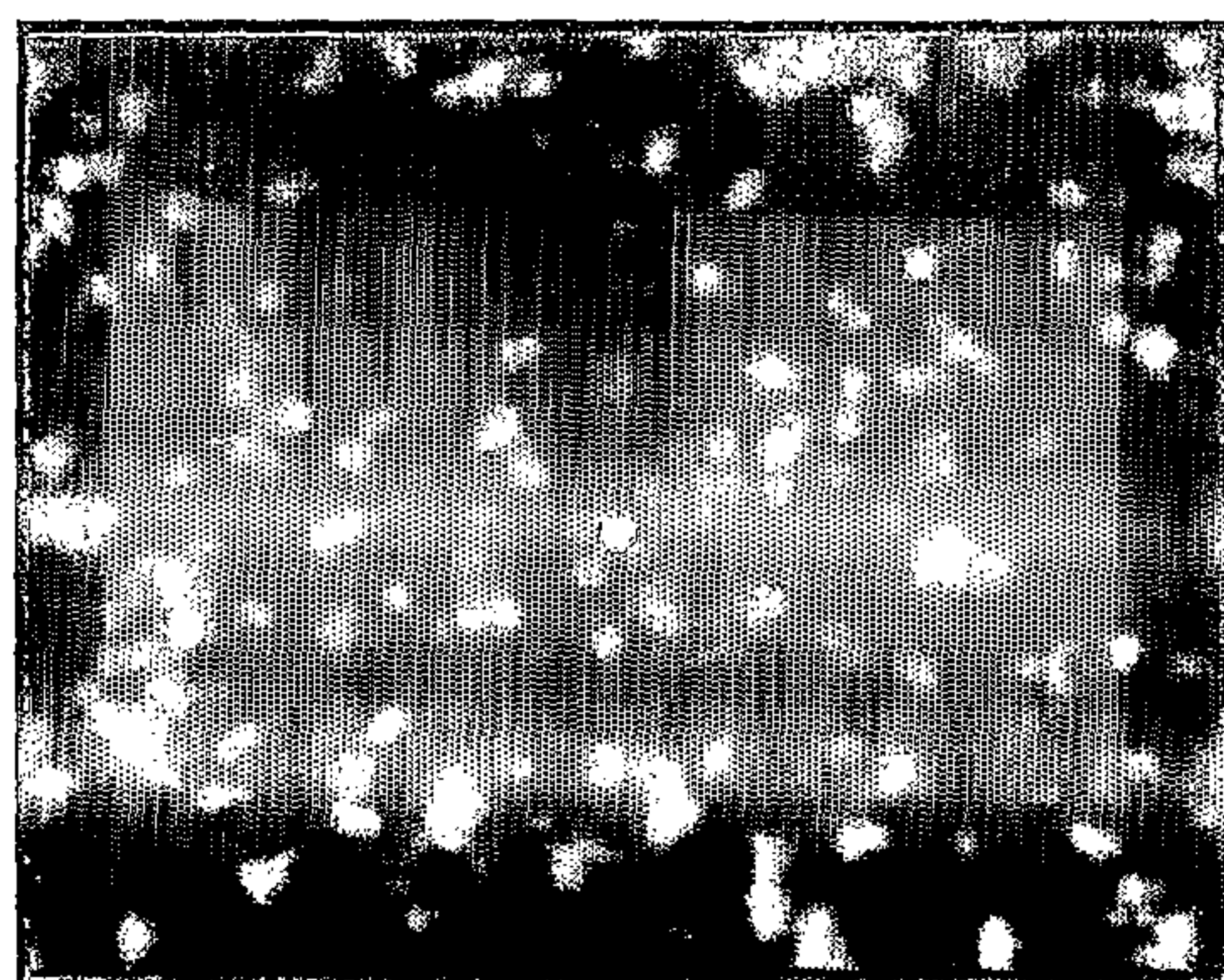
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*FIG. 13D**FIG. 14**FIG. 15*

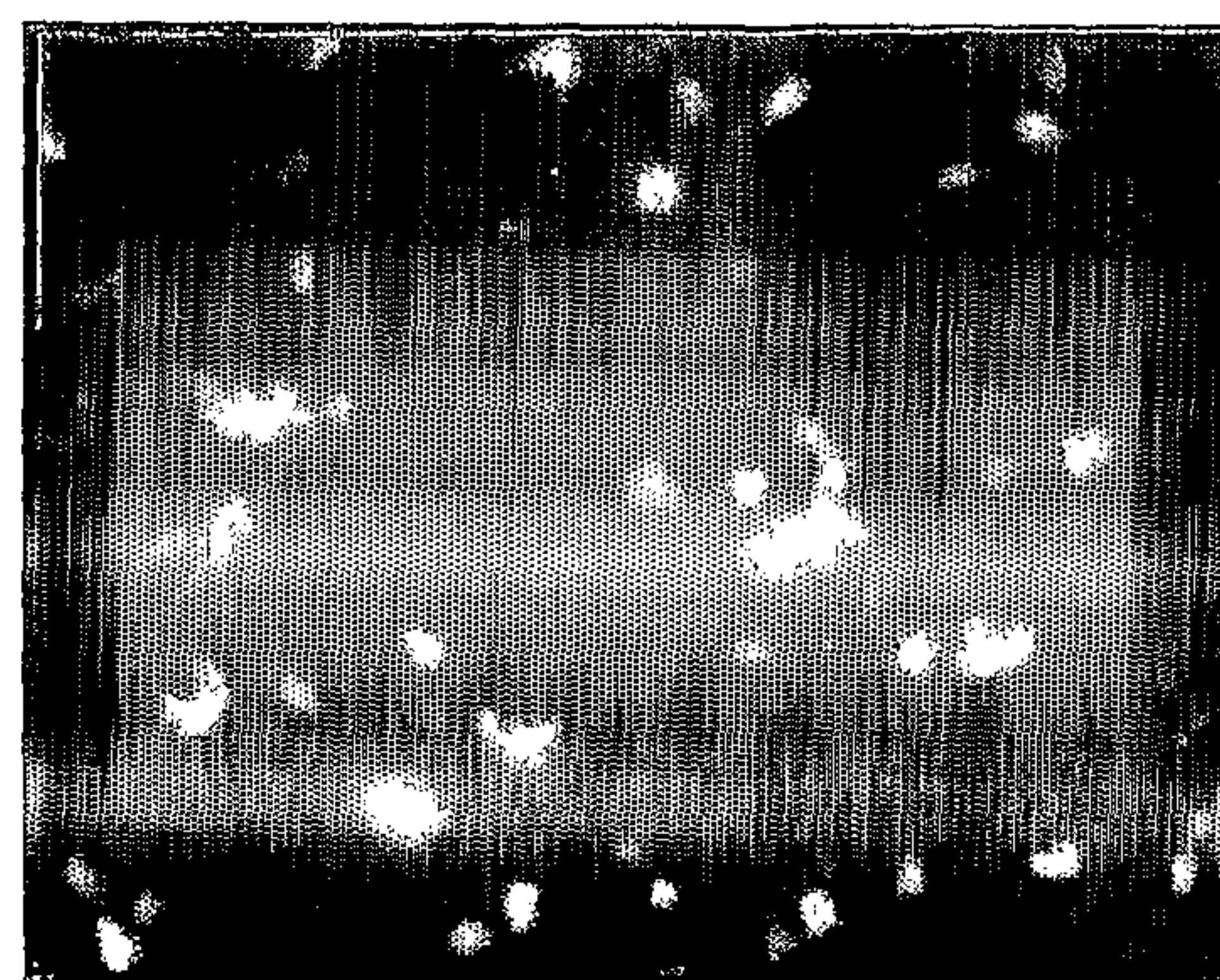
pIM45/pIM45VP0

FIG. 16A

pIM45VP1/pIM45VP2,3

FIG. 16B

pIM45VP2acg/atg/pIM45VP1,3

FIG. 16C

pIM45VP3/pIM45VP1,2

FIG. 16D

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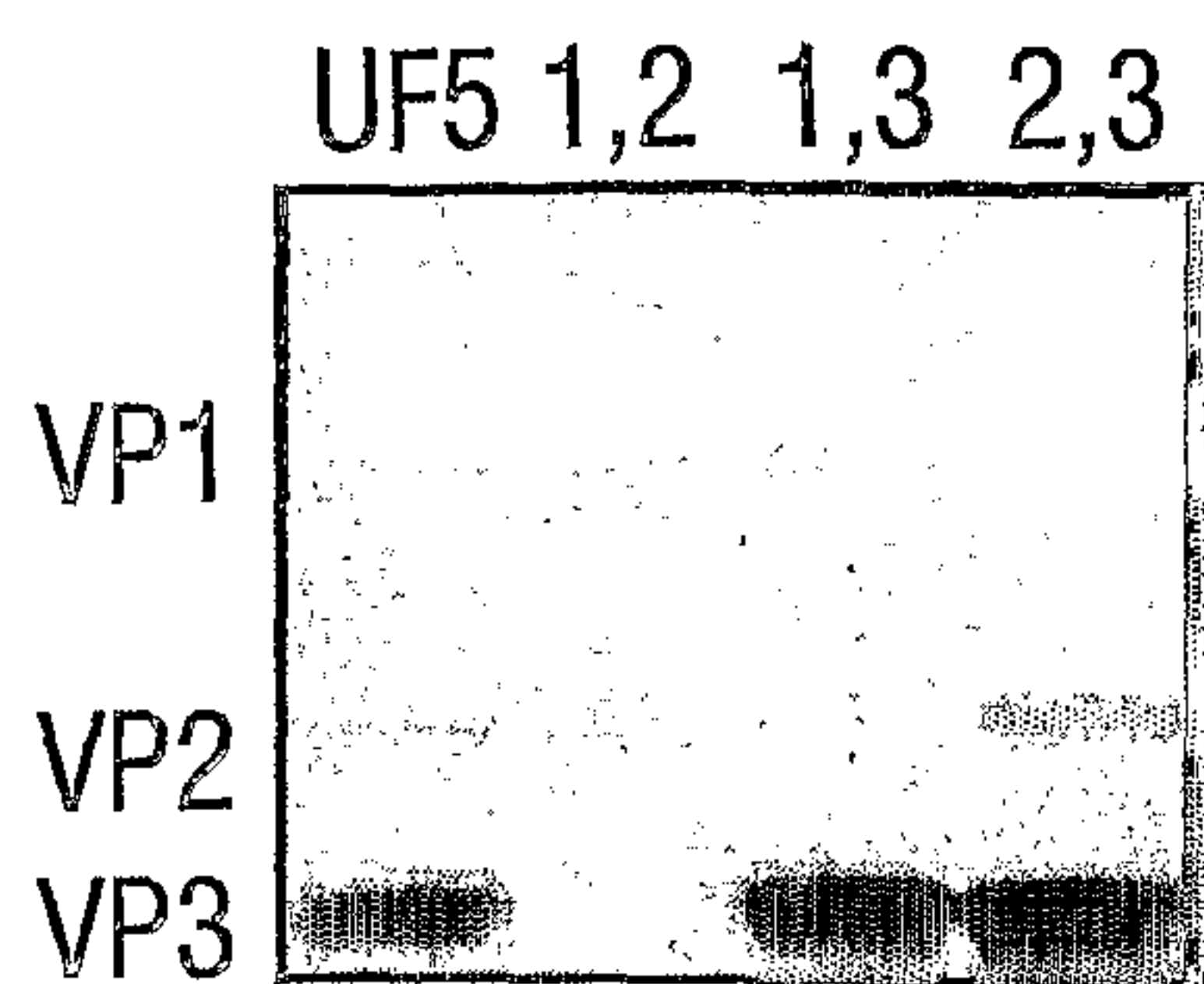


FIG. 17A

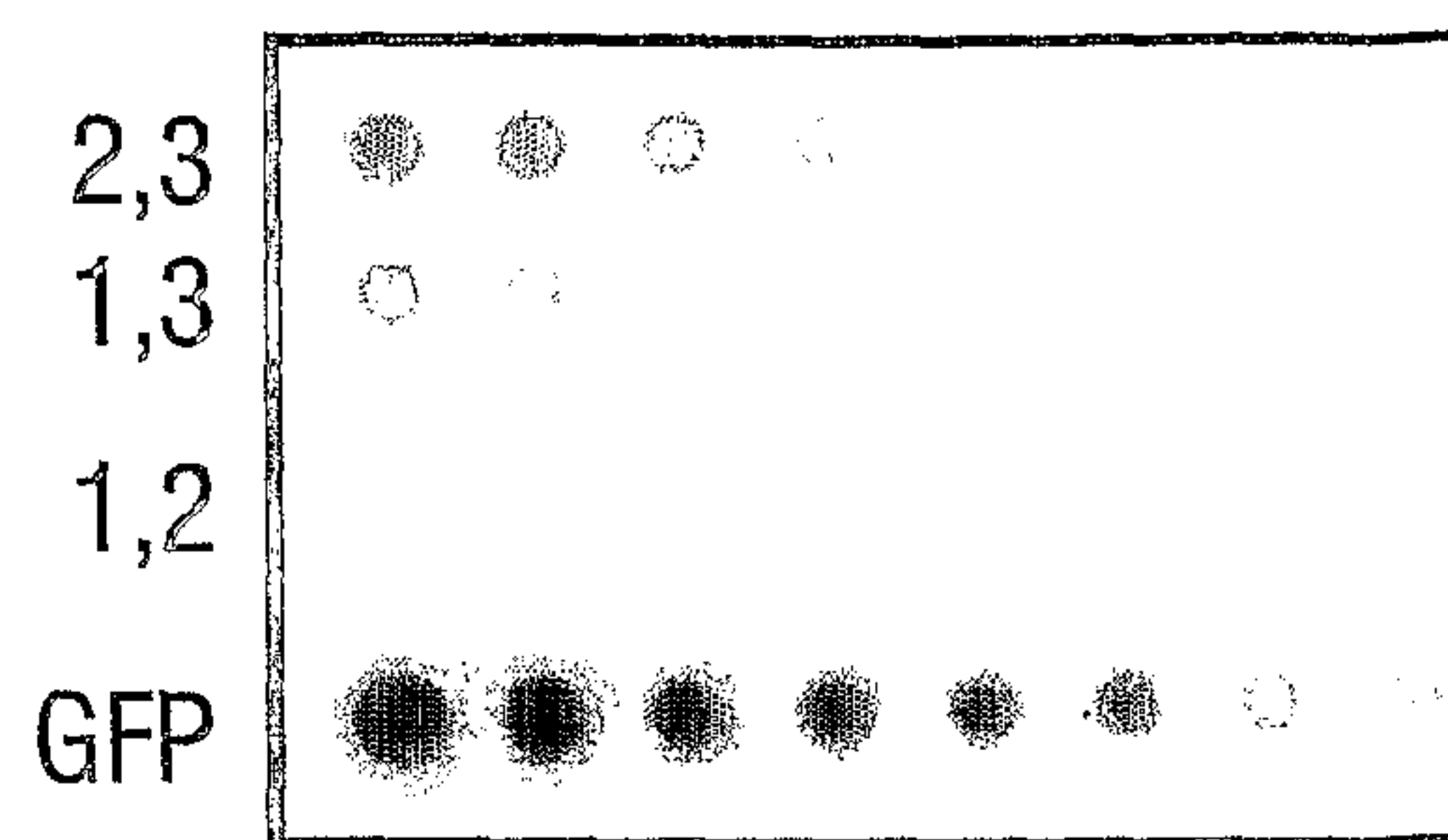
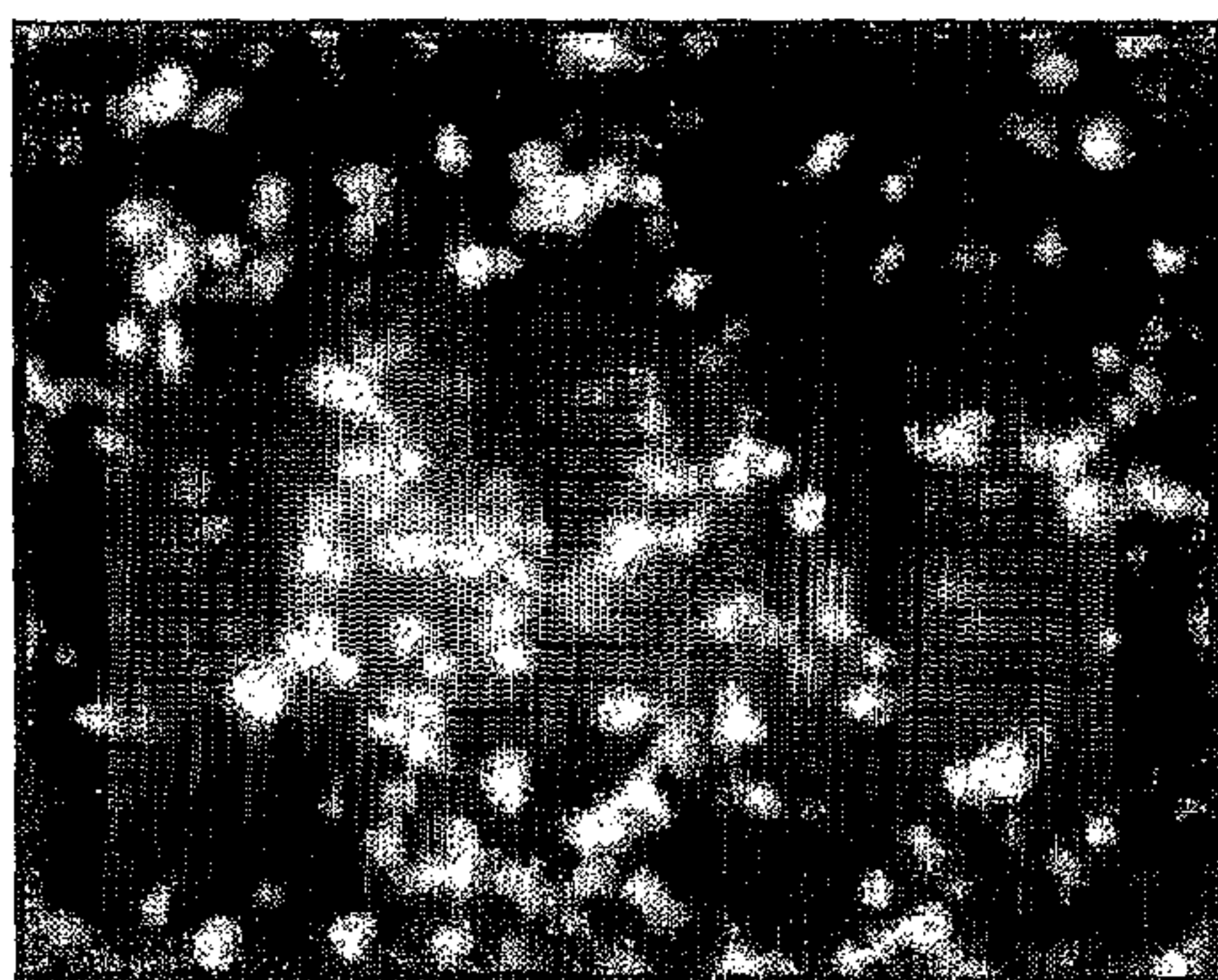
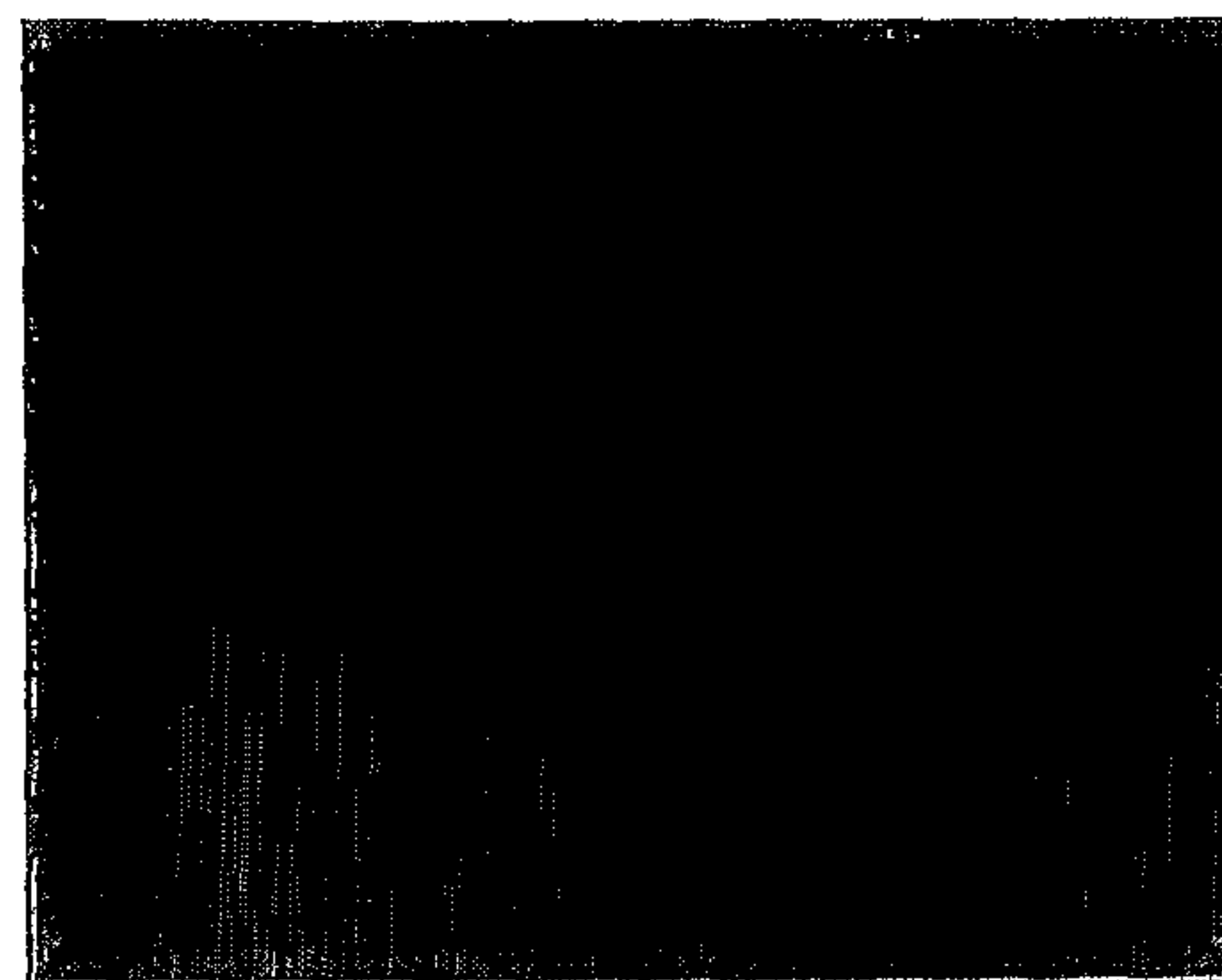


FIG. 17B



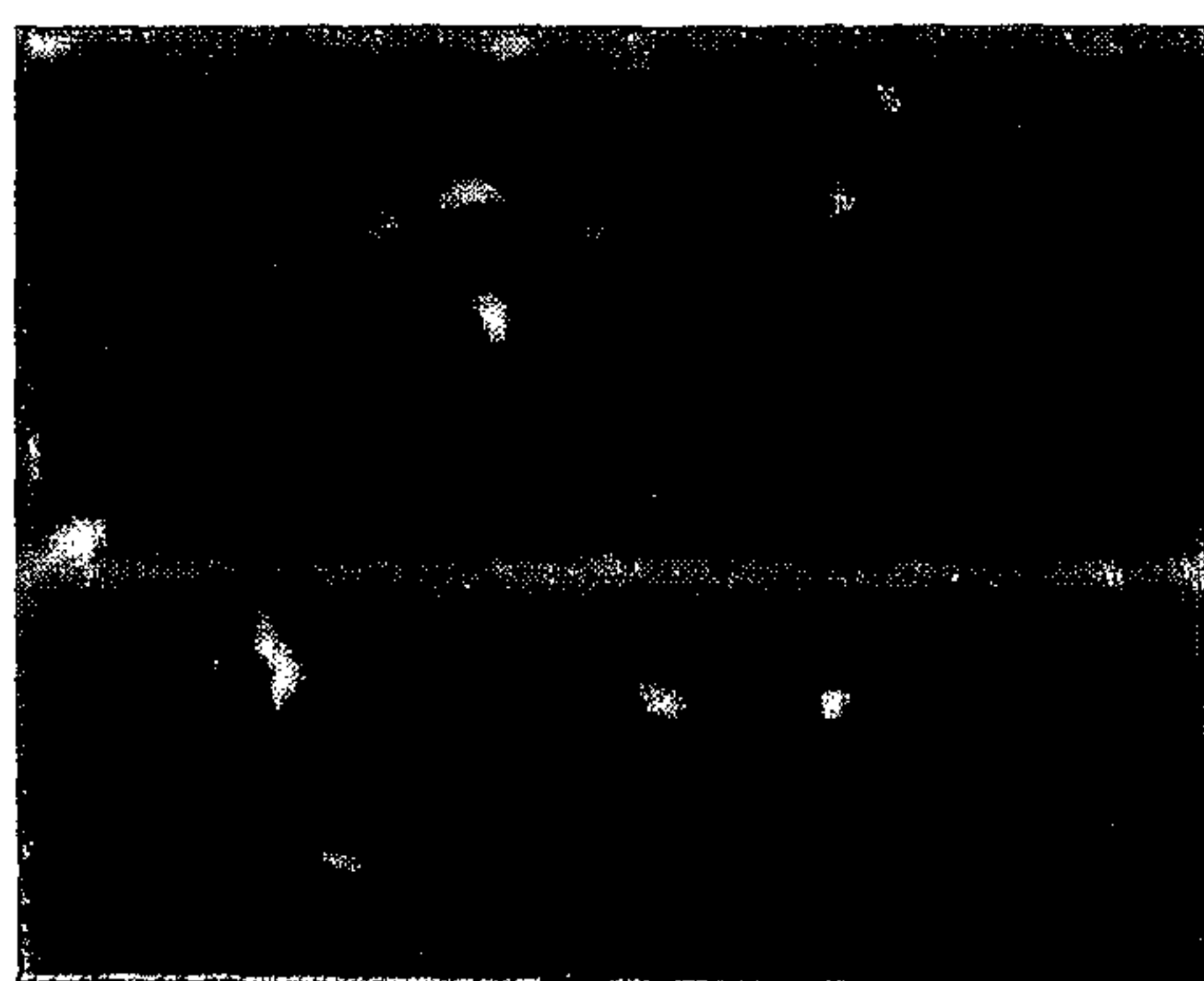
UF5

FIG. 18A



1,2

FIG. 18B



1,3

FIG. 18C



2,3

FIG. 18D

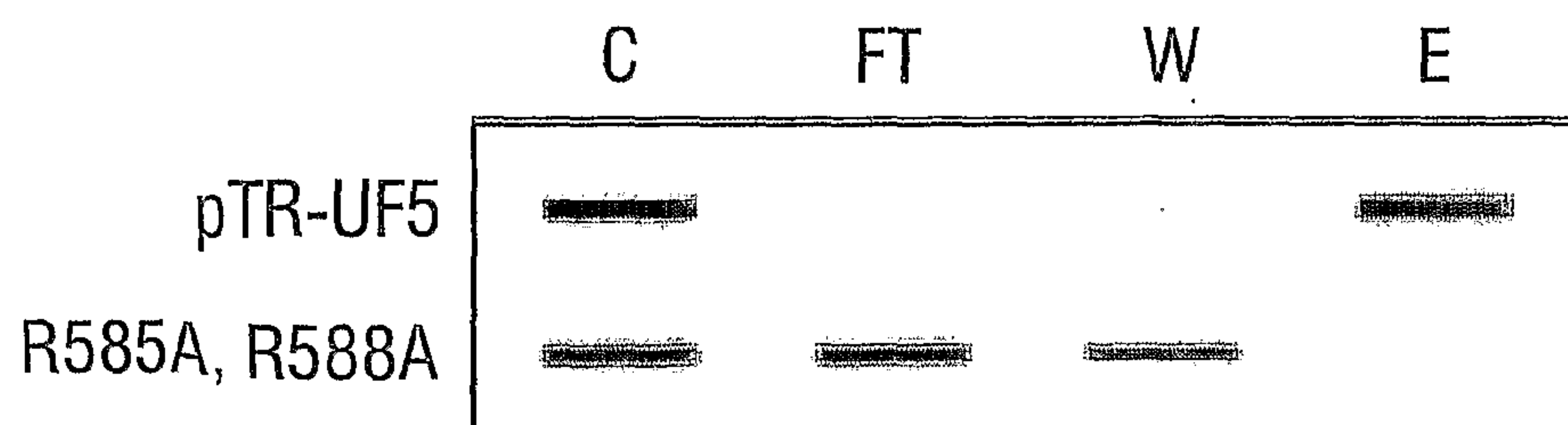


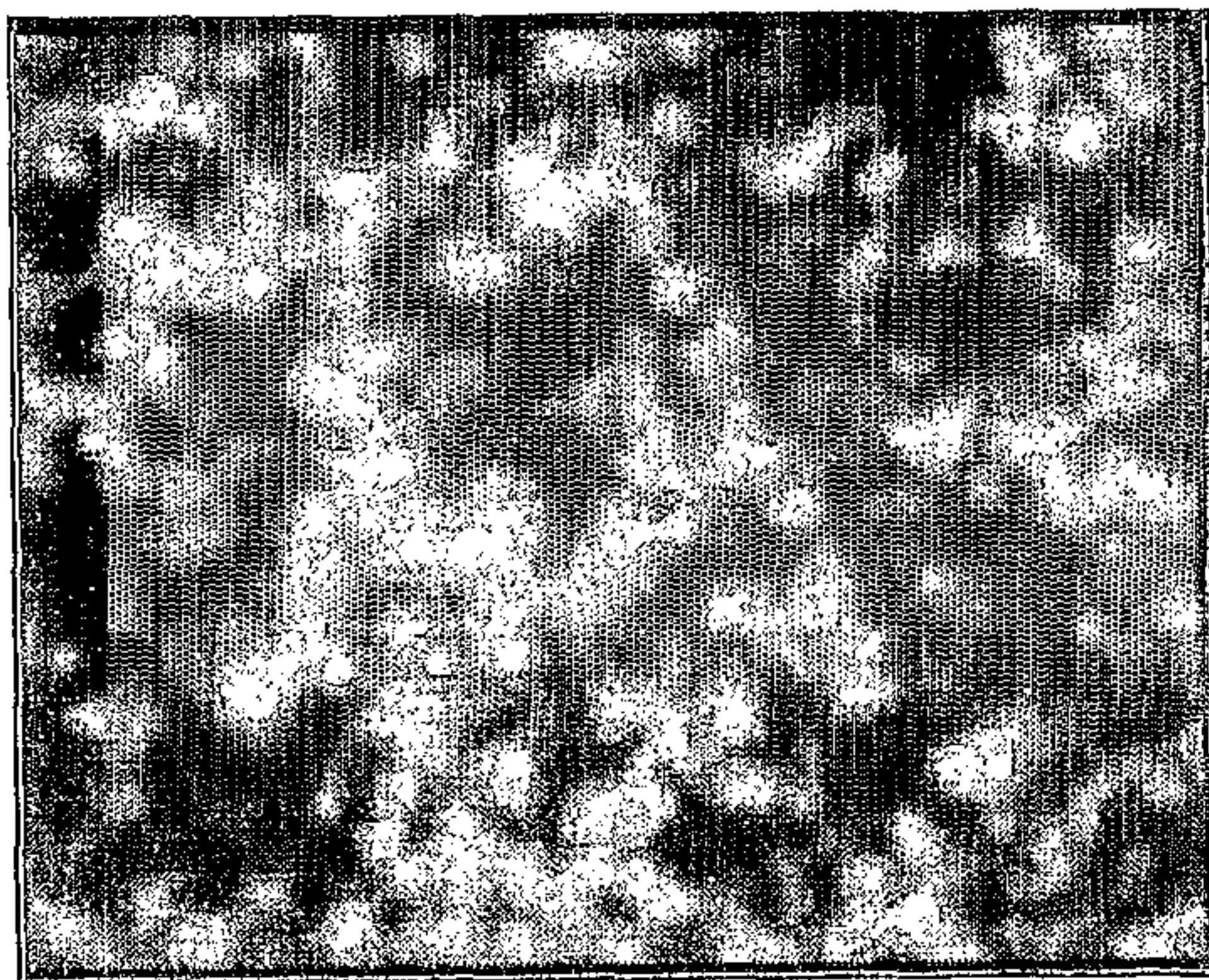
FIG. 19

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pTR-UF5
R585A, R588A

*FIG. 20*

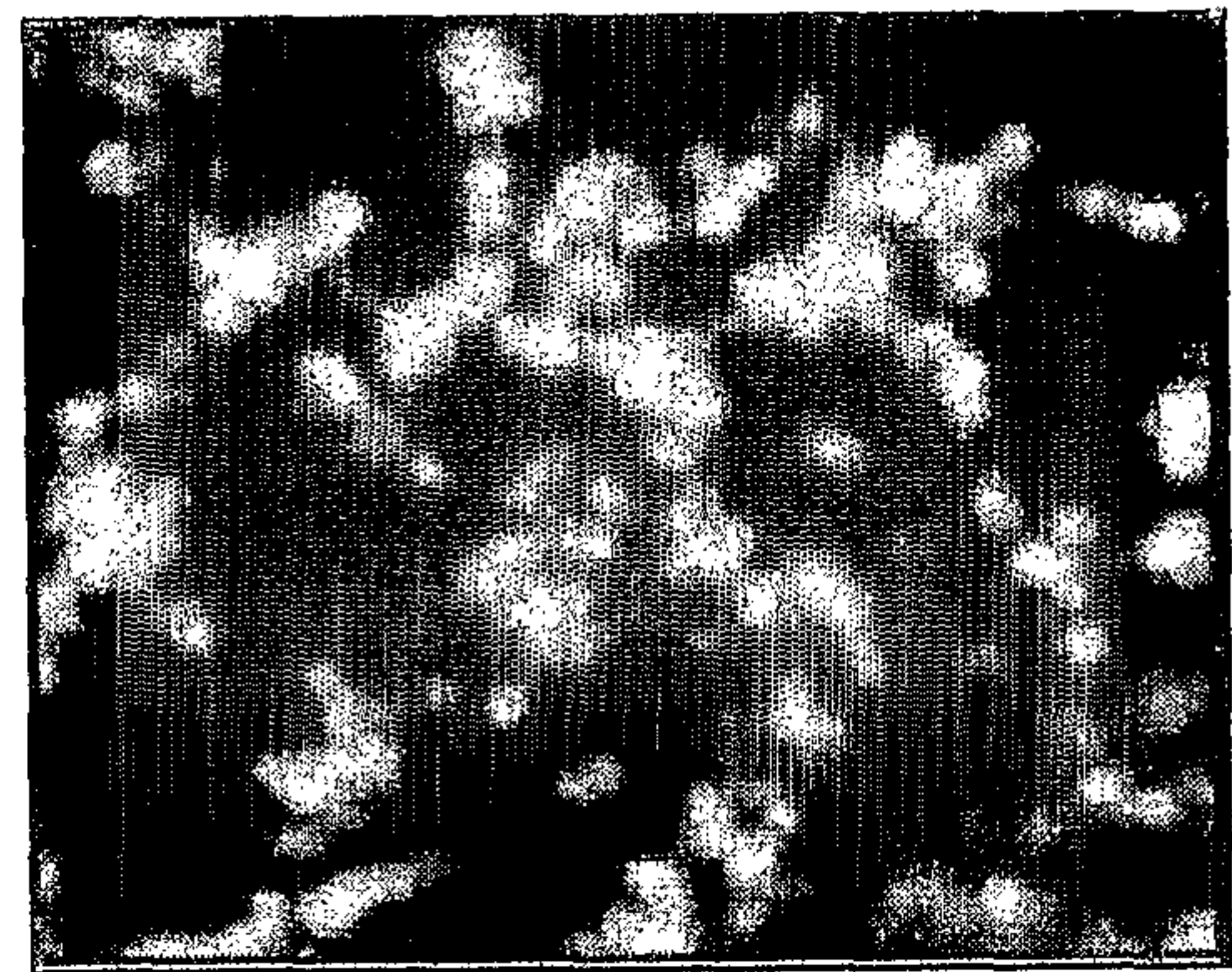
pTR-UF5



HeLaC12

FIG. 21A

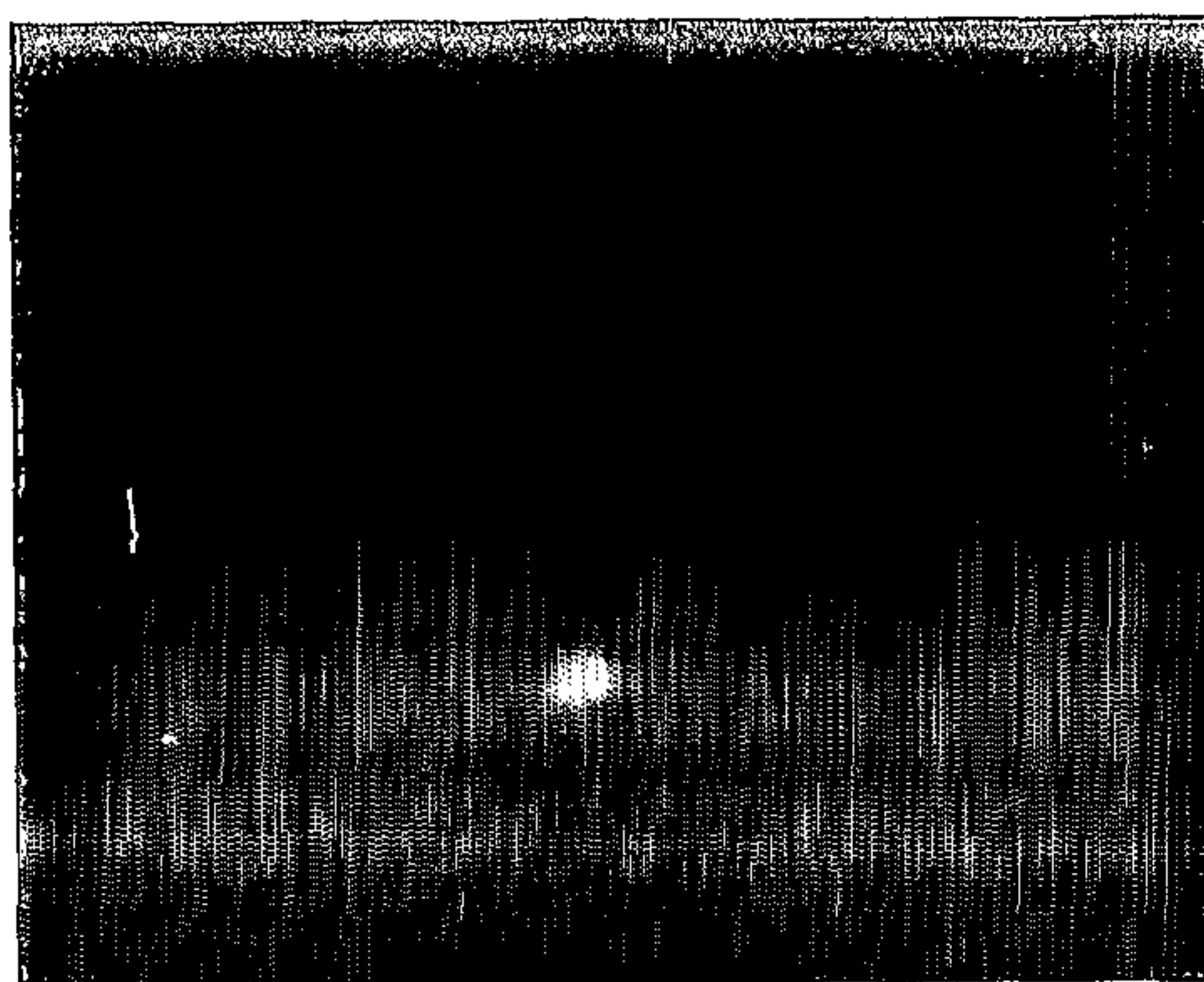
pTR-UF5



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FIG. 21B

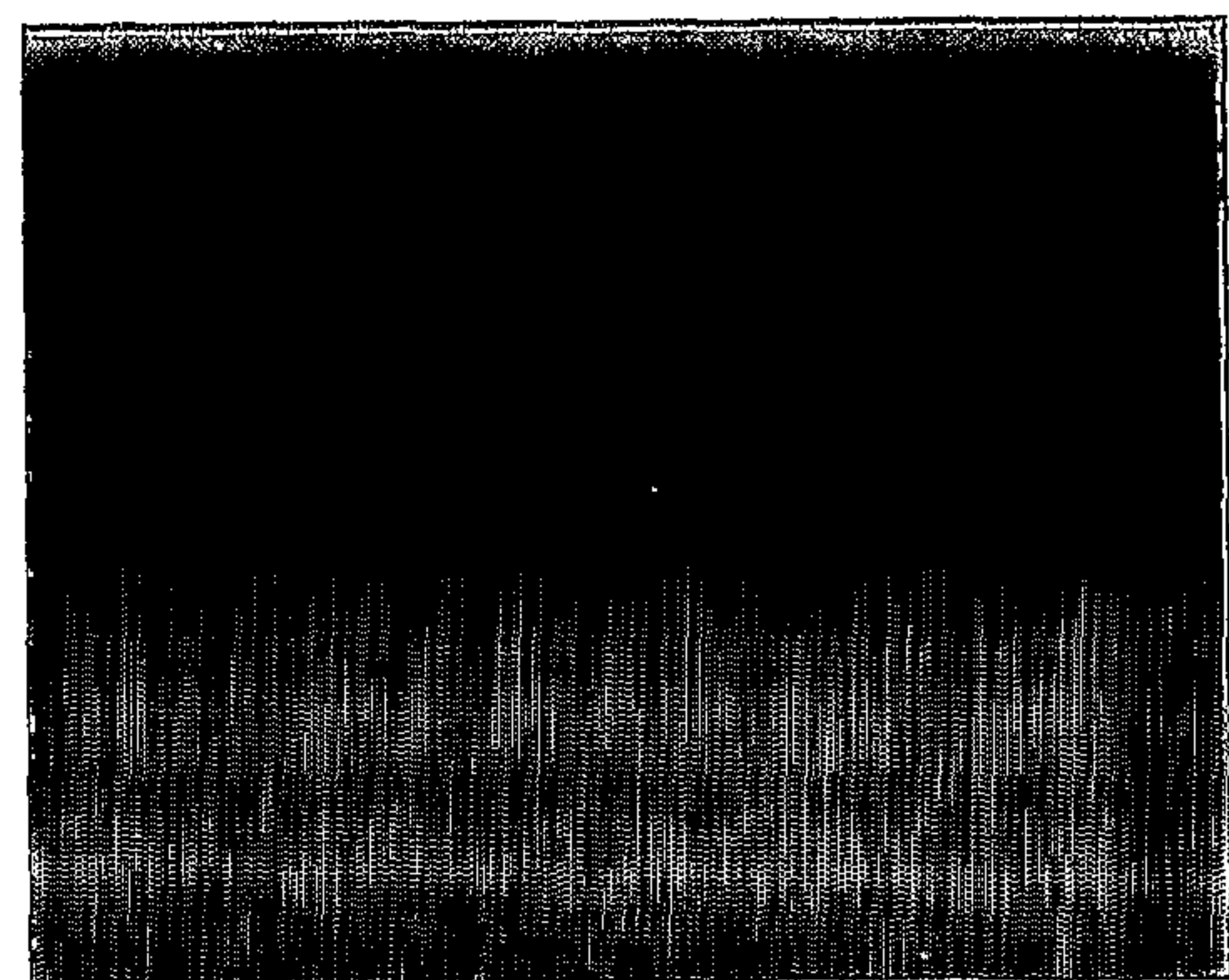
R585A, R588A



HeLaC12

FIG. 21C

R585A, R588A



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FIG. 21D

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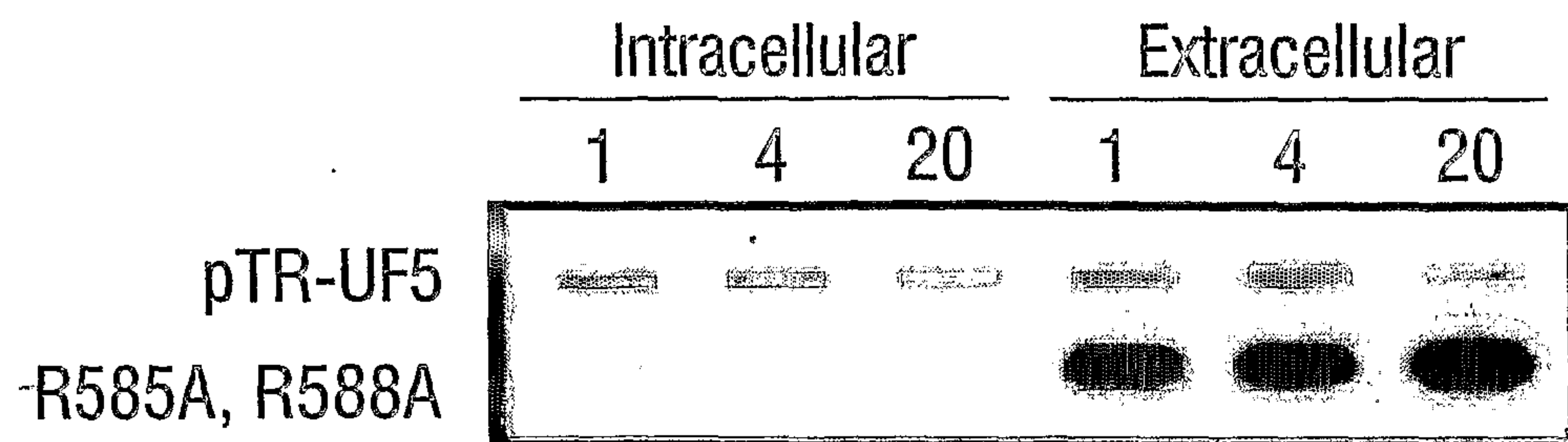


FIG. 22

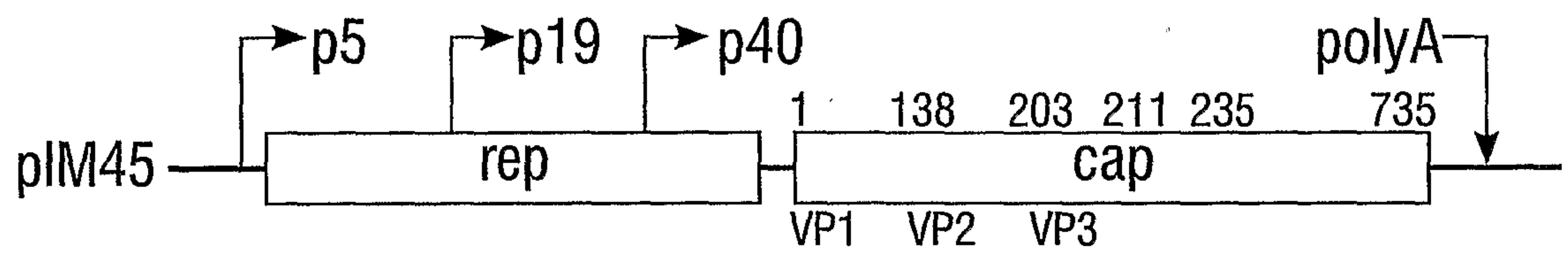
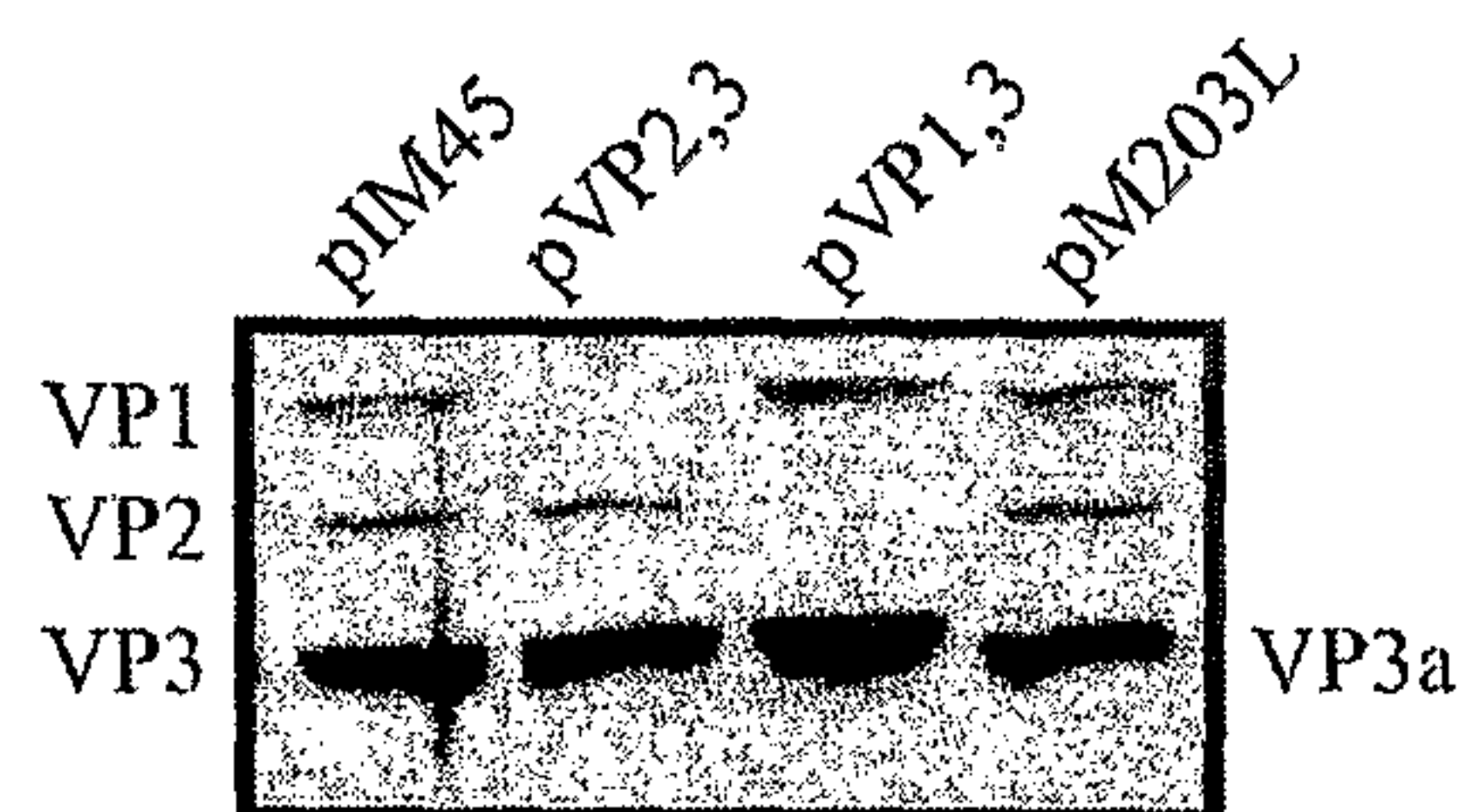
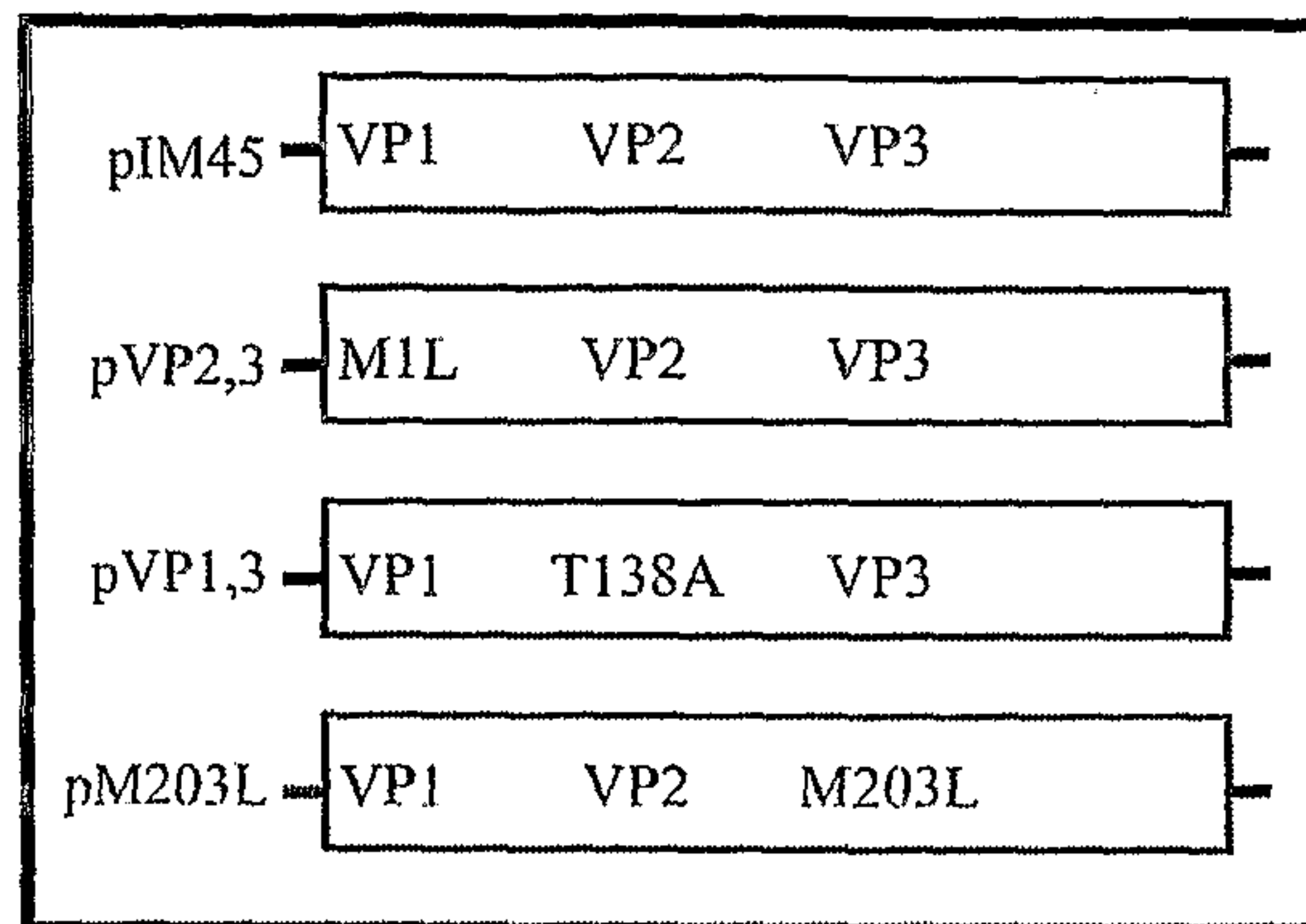


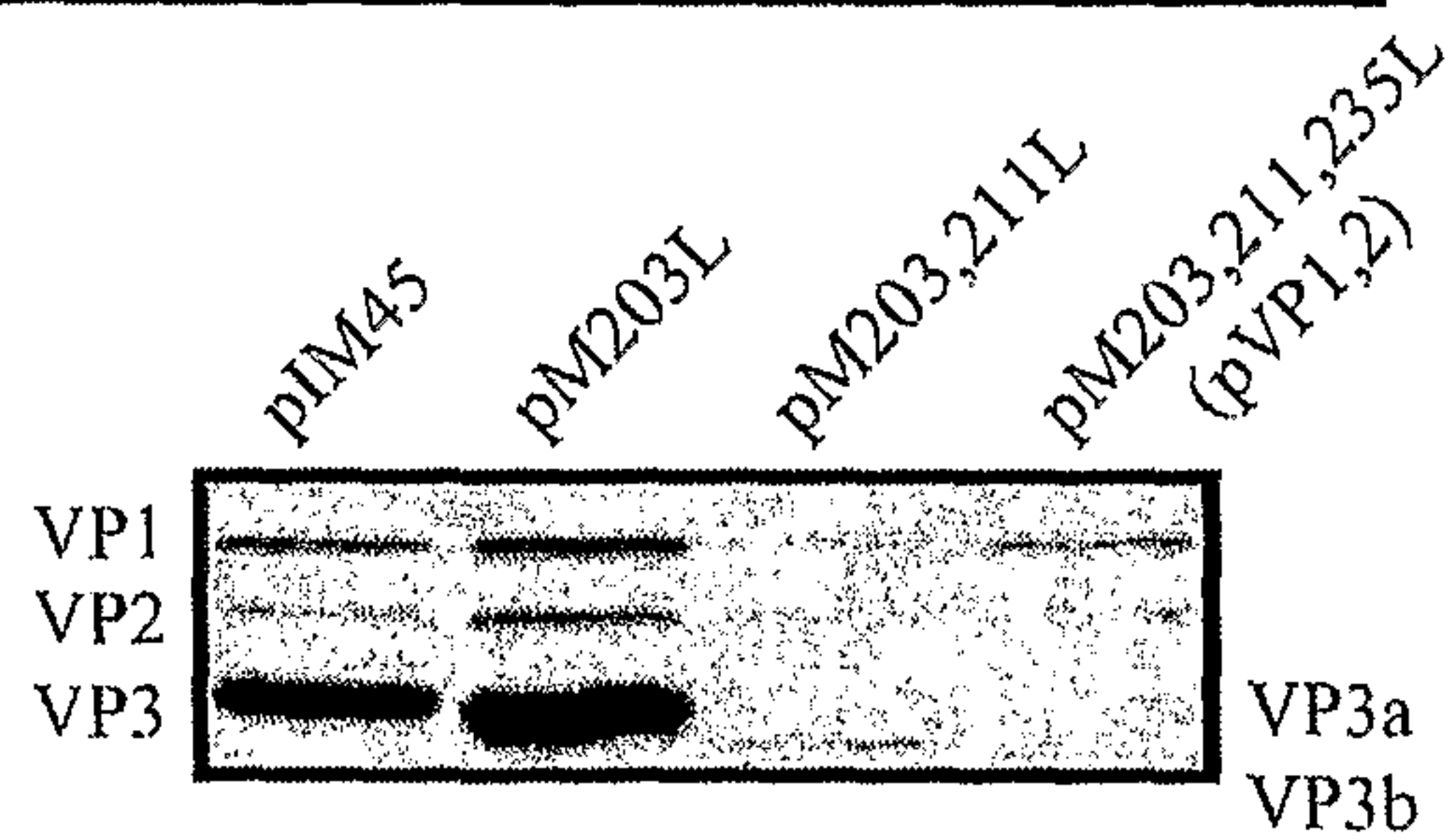
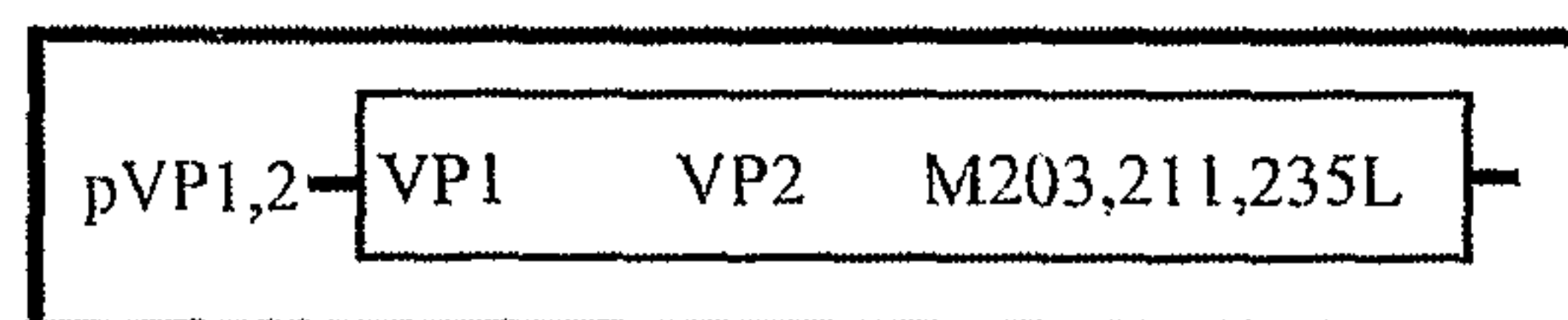
FIG. 23

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A



B



C

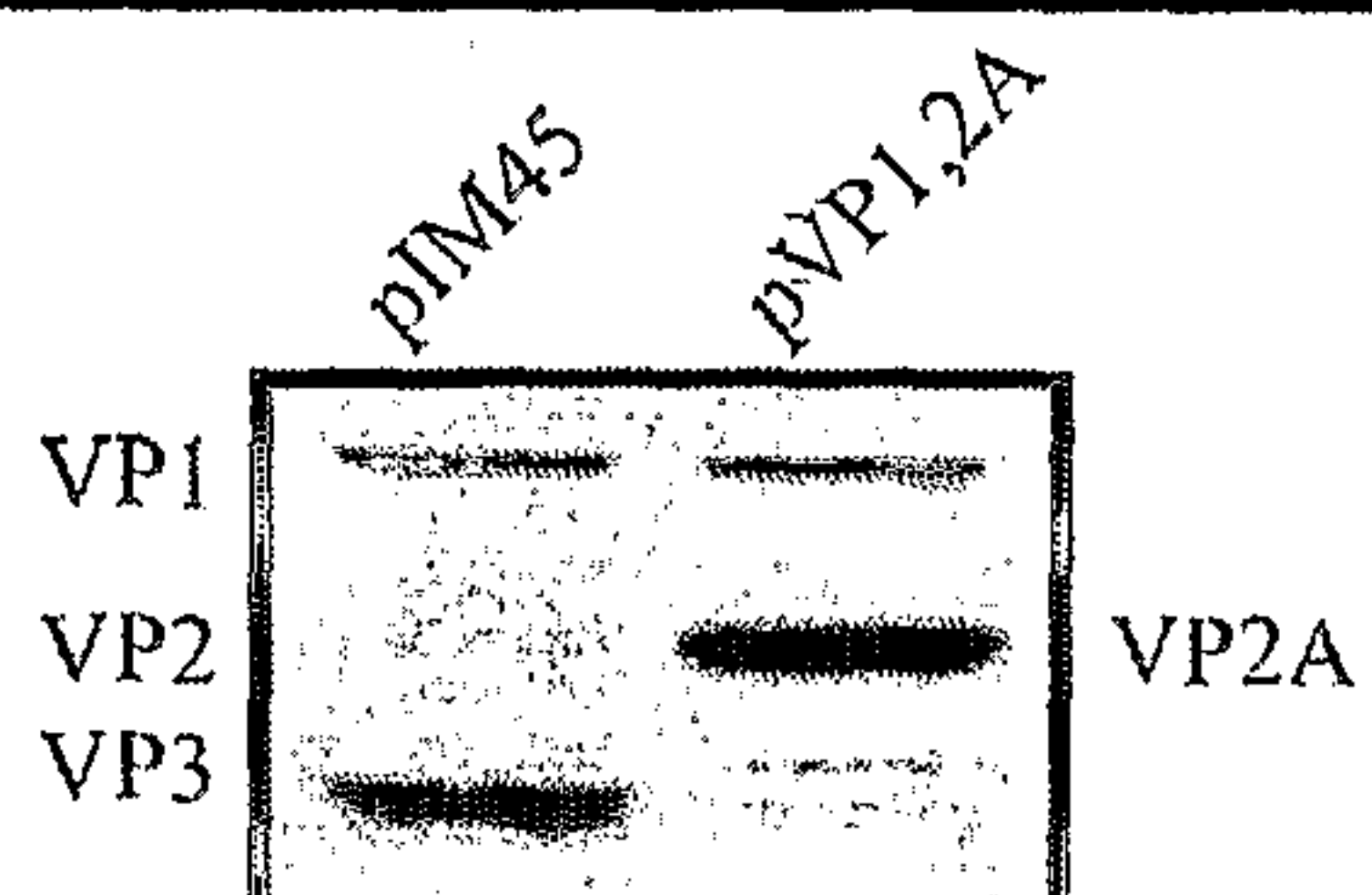
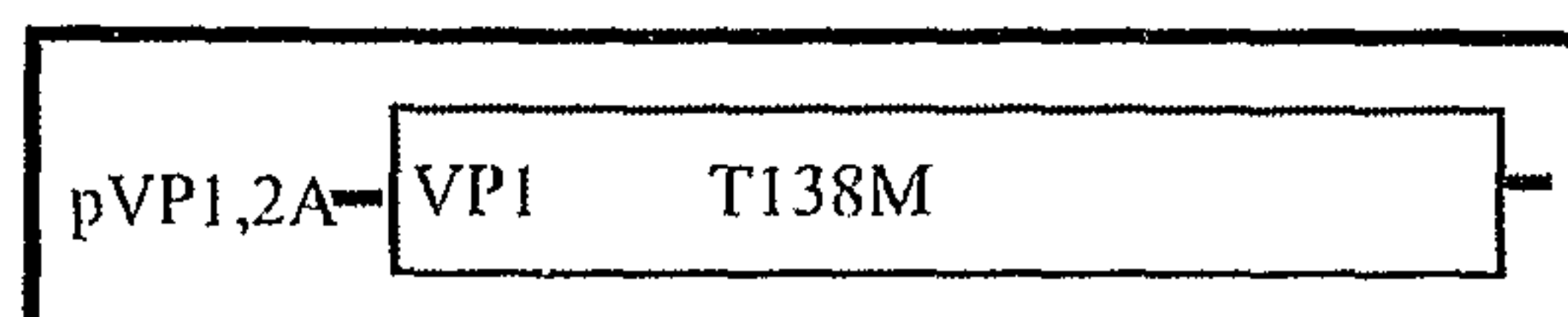


FIG. 25

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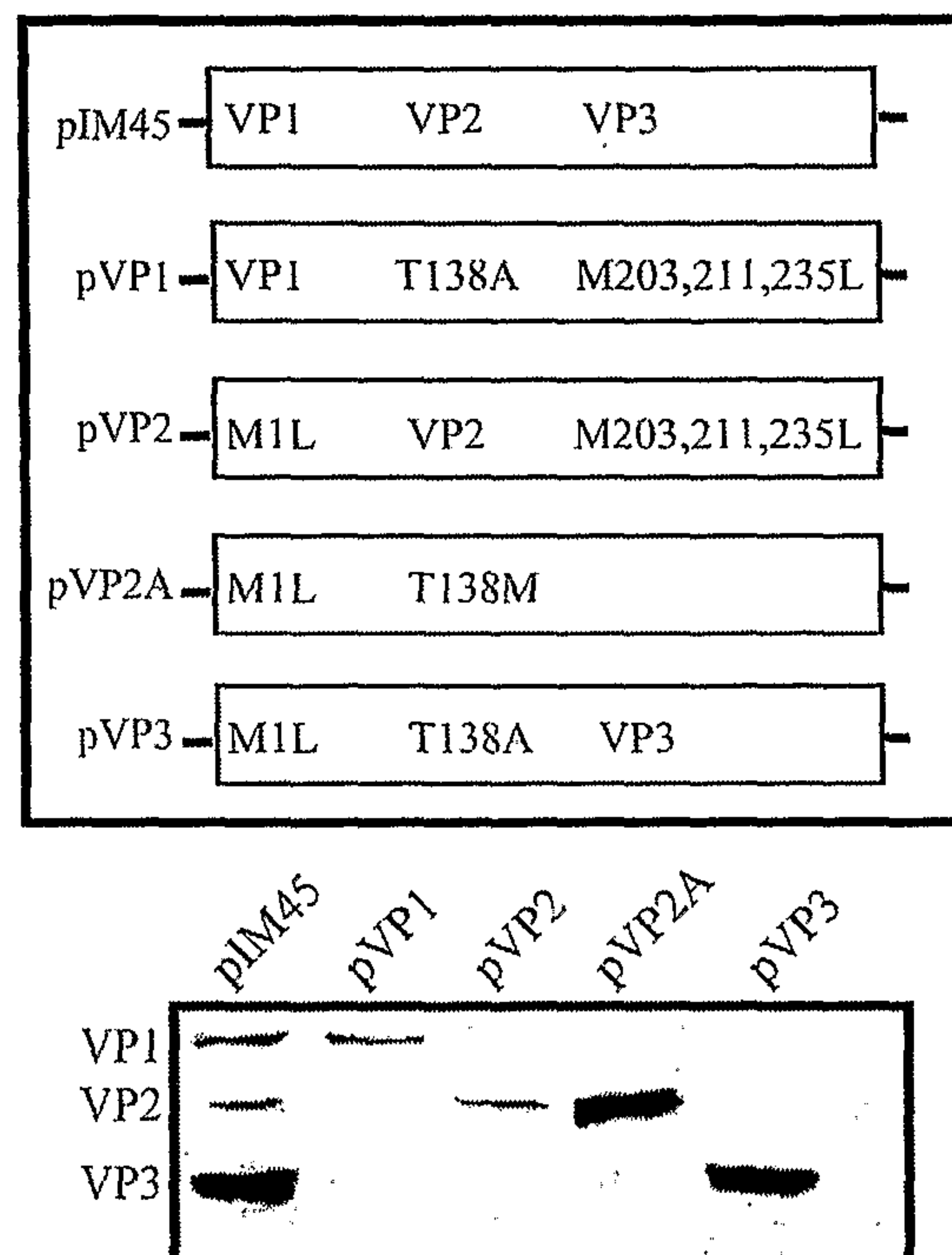
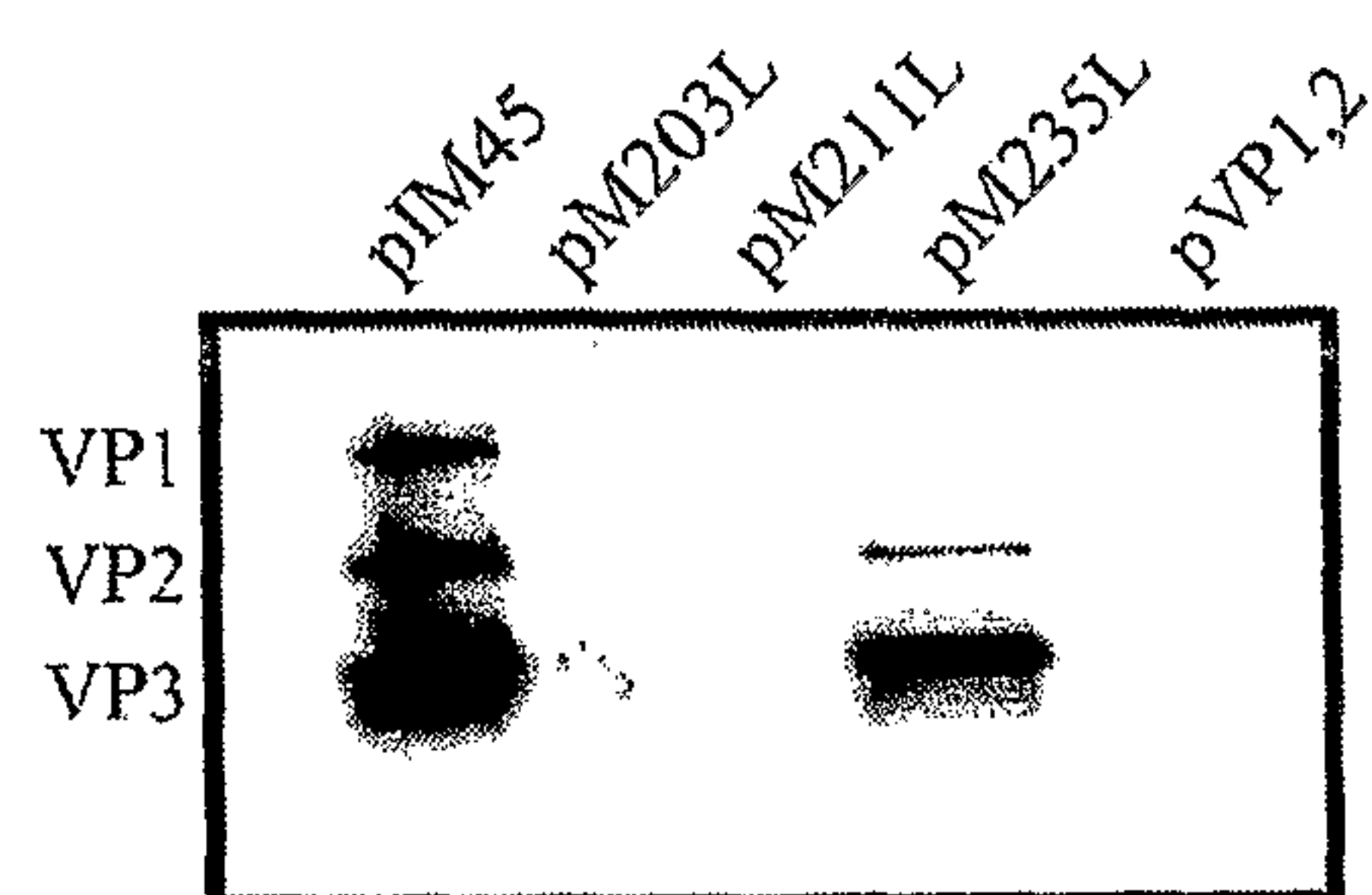


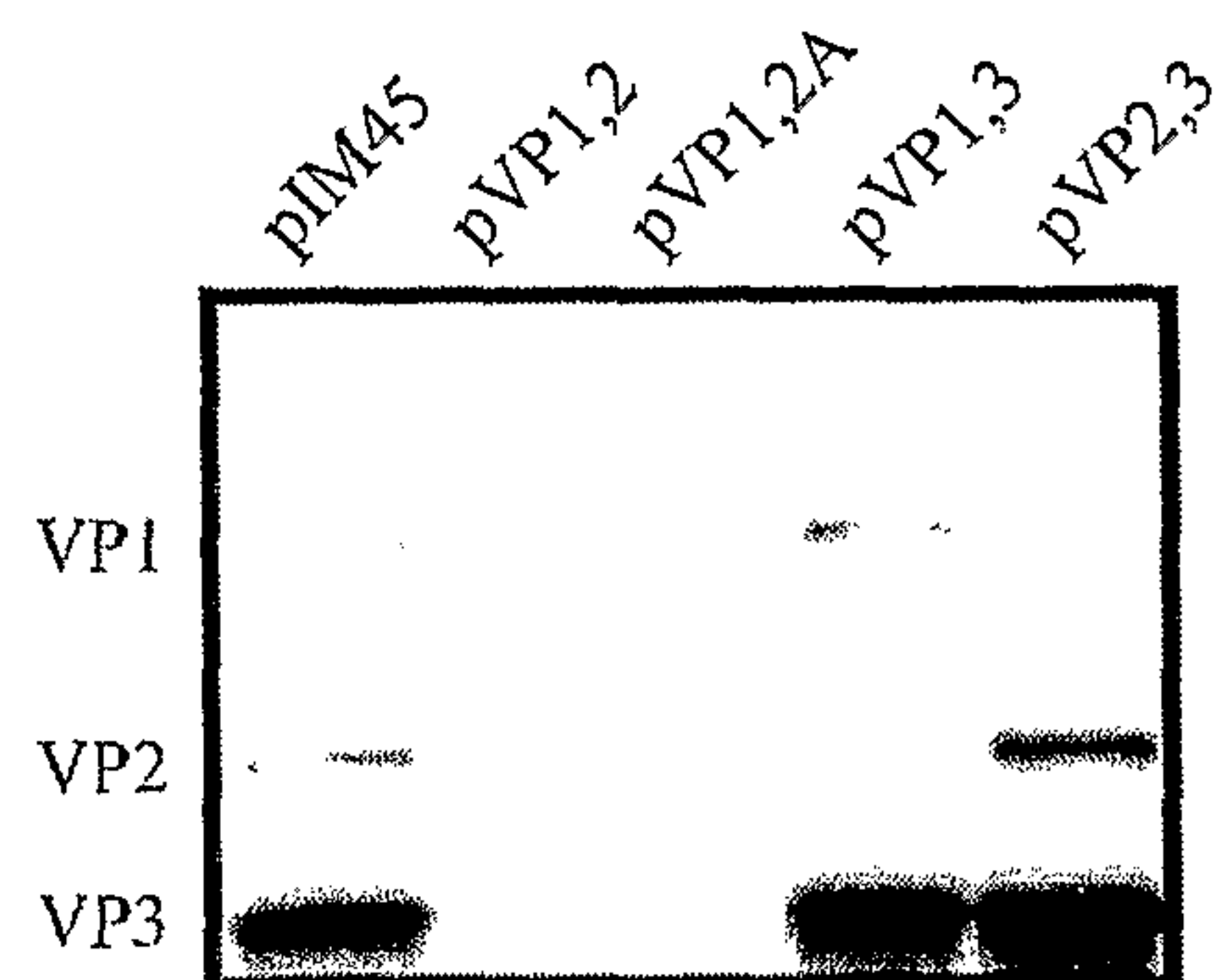
FIG. 26

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A



B



C

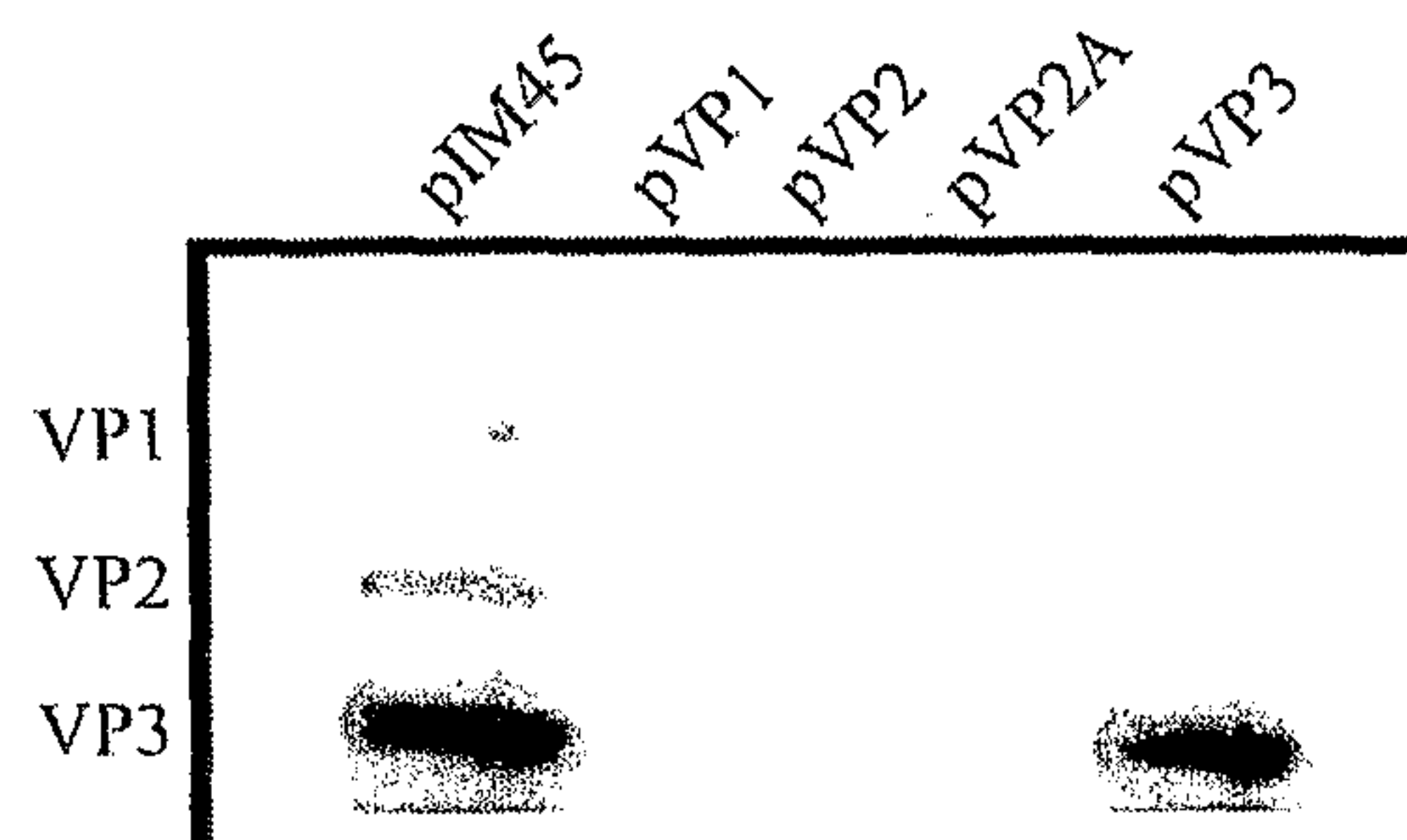


FIG. 27

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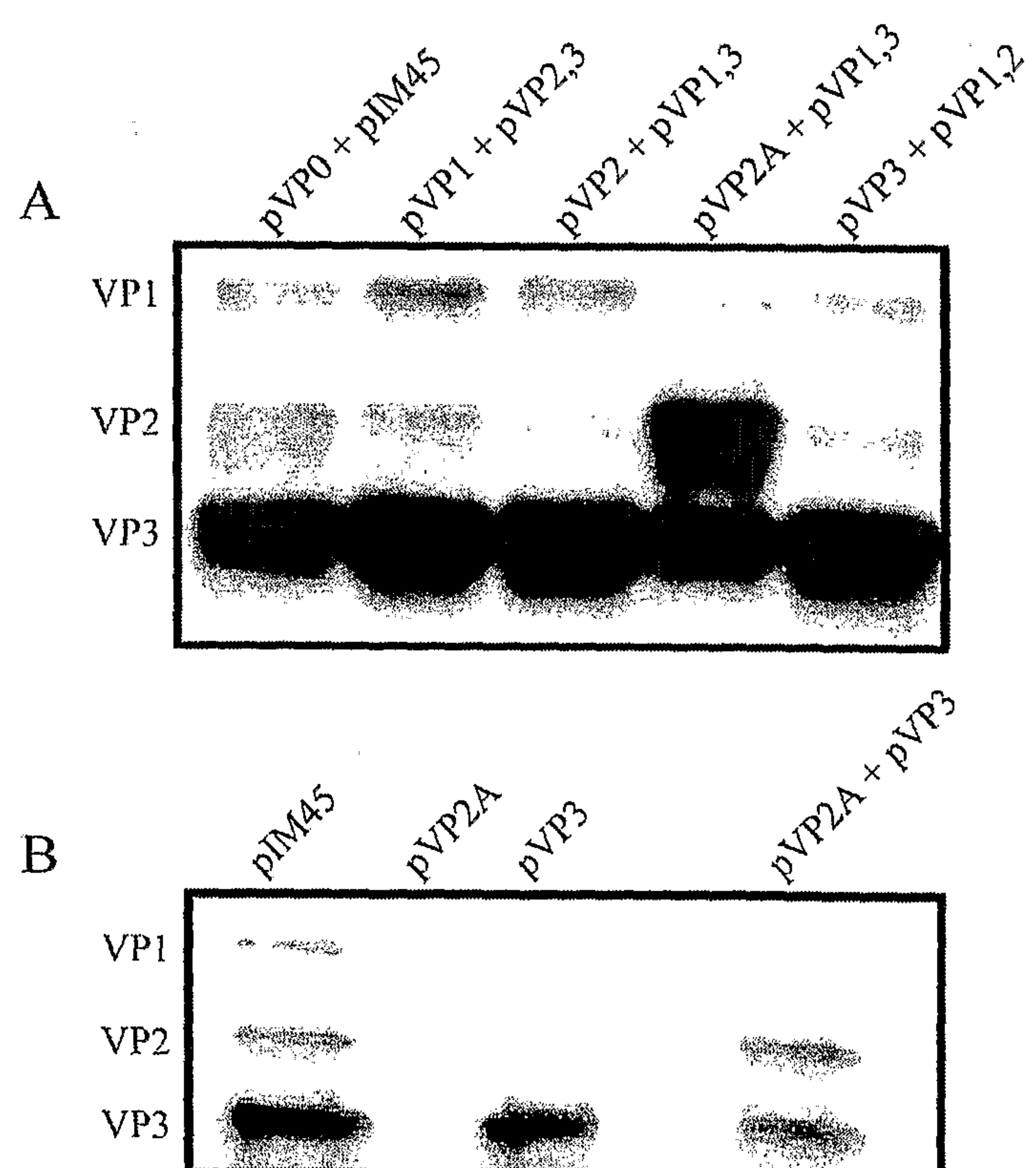


FIG. 28

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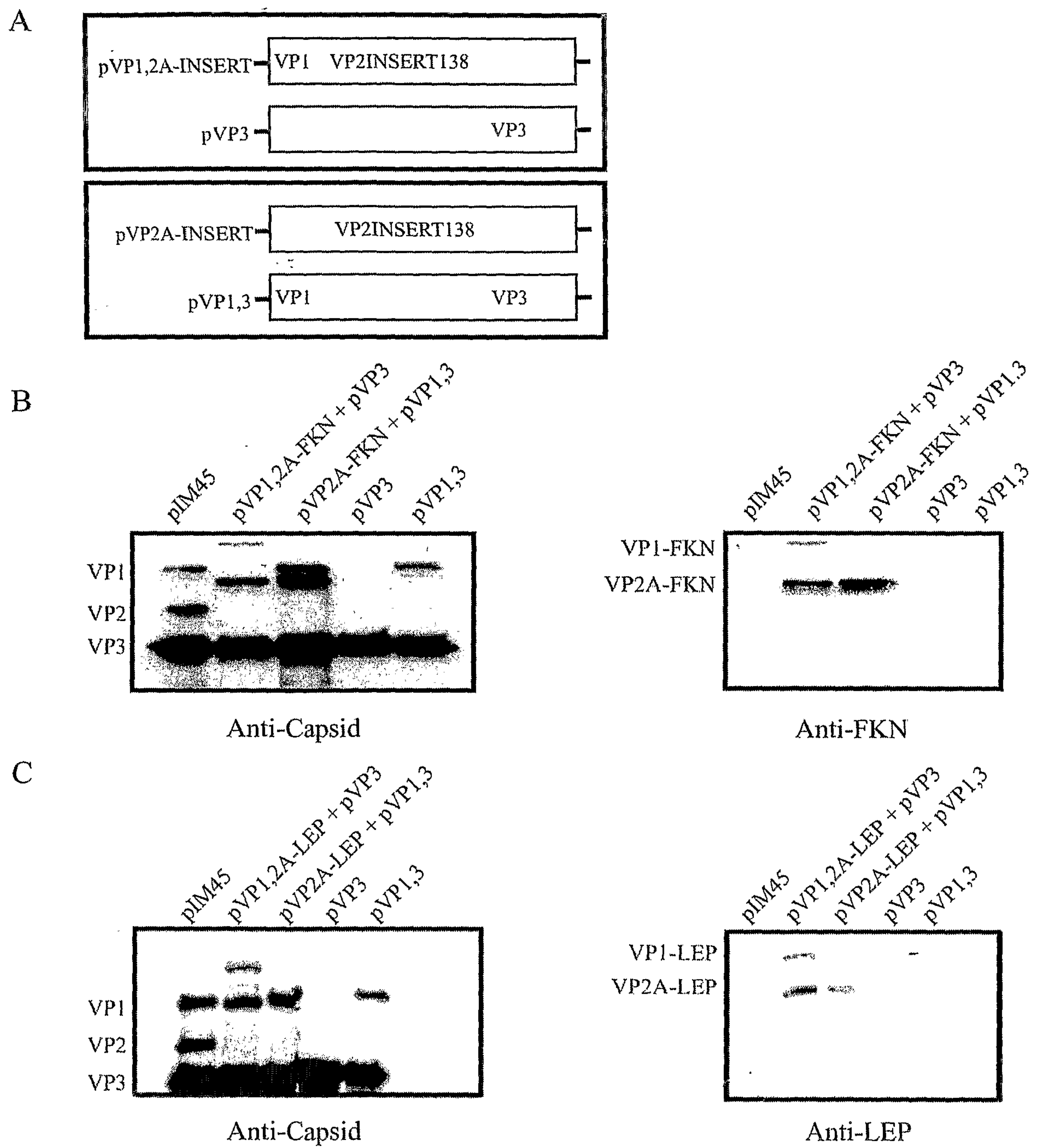


FIG. 29

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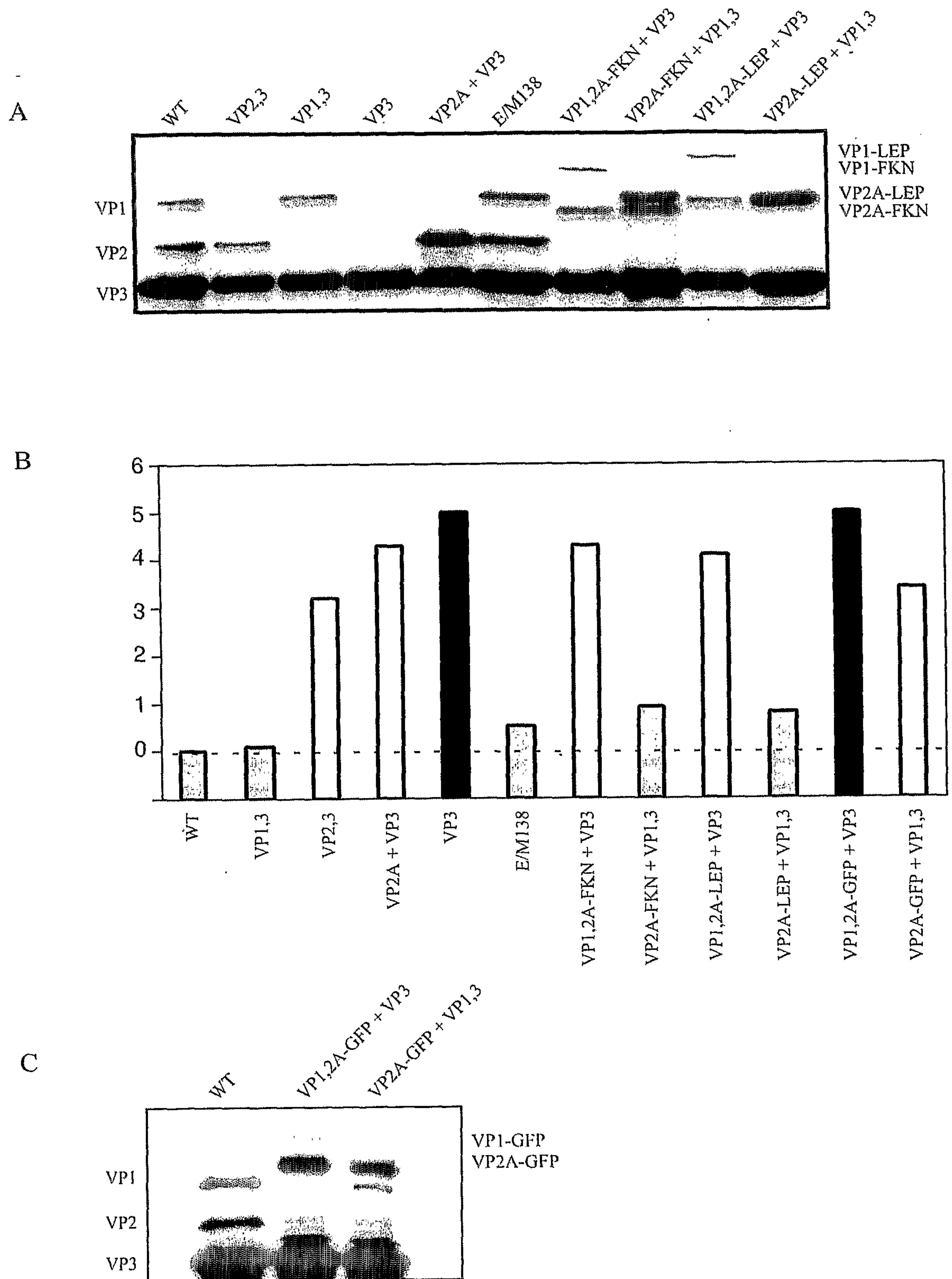


FIG. 30

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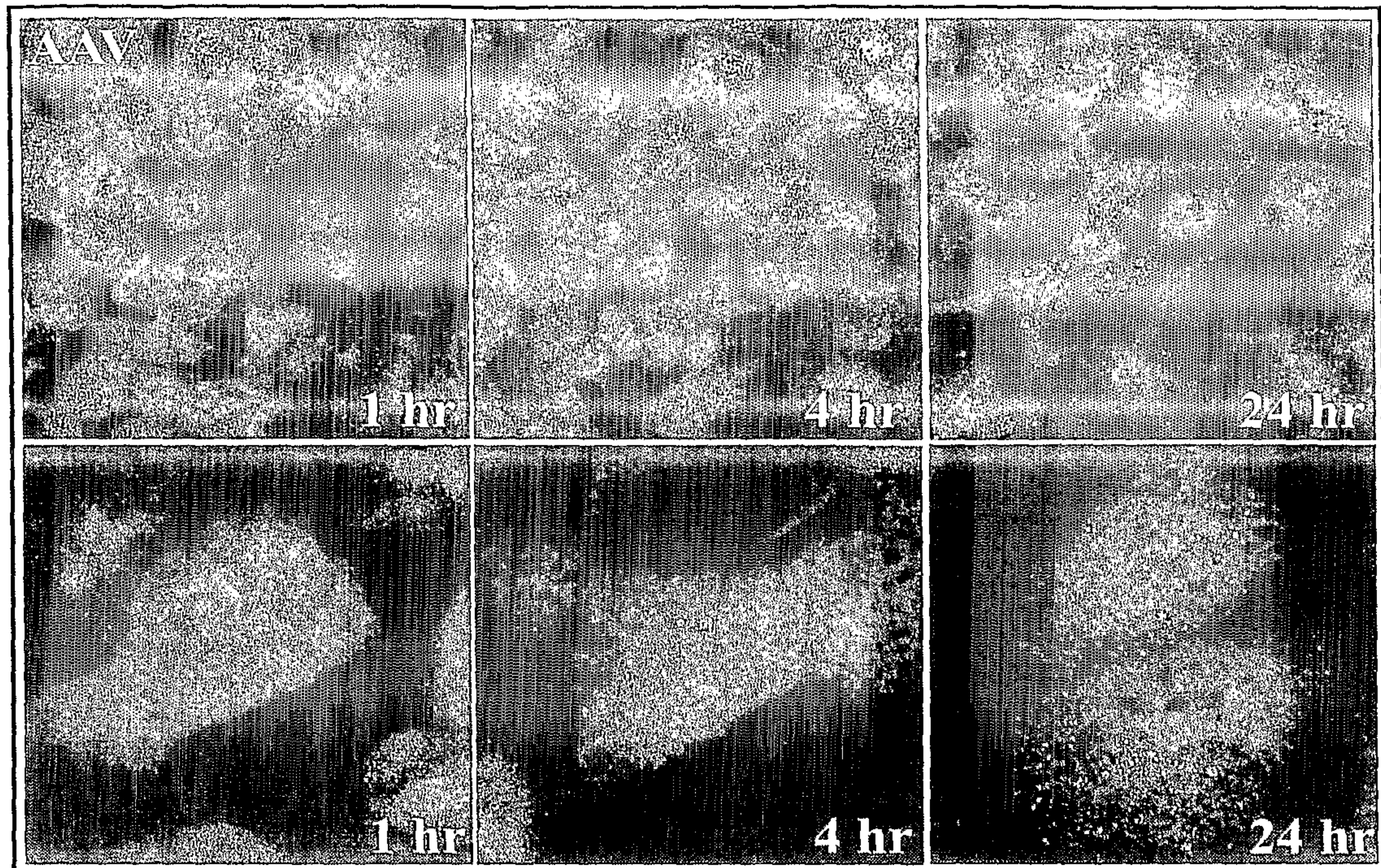


FIG. 31A

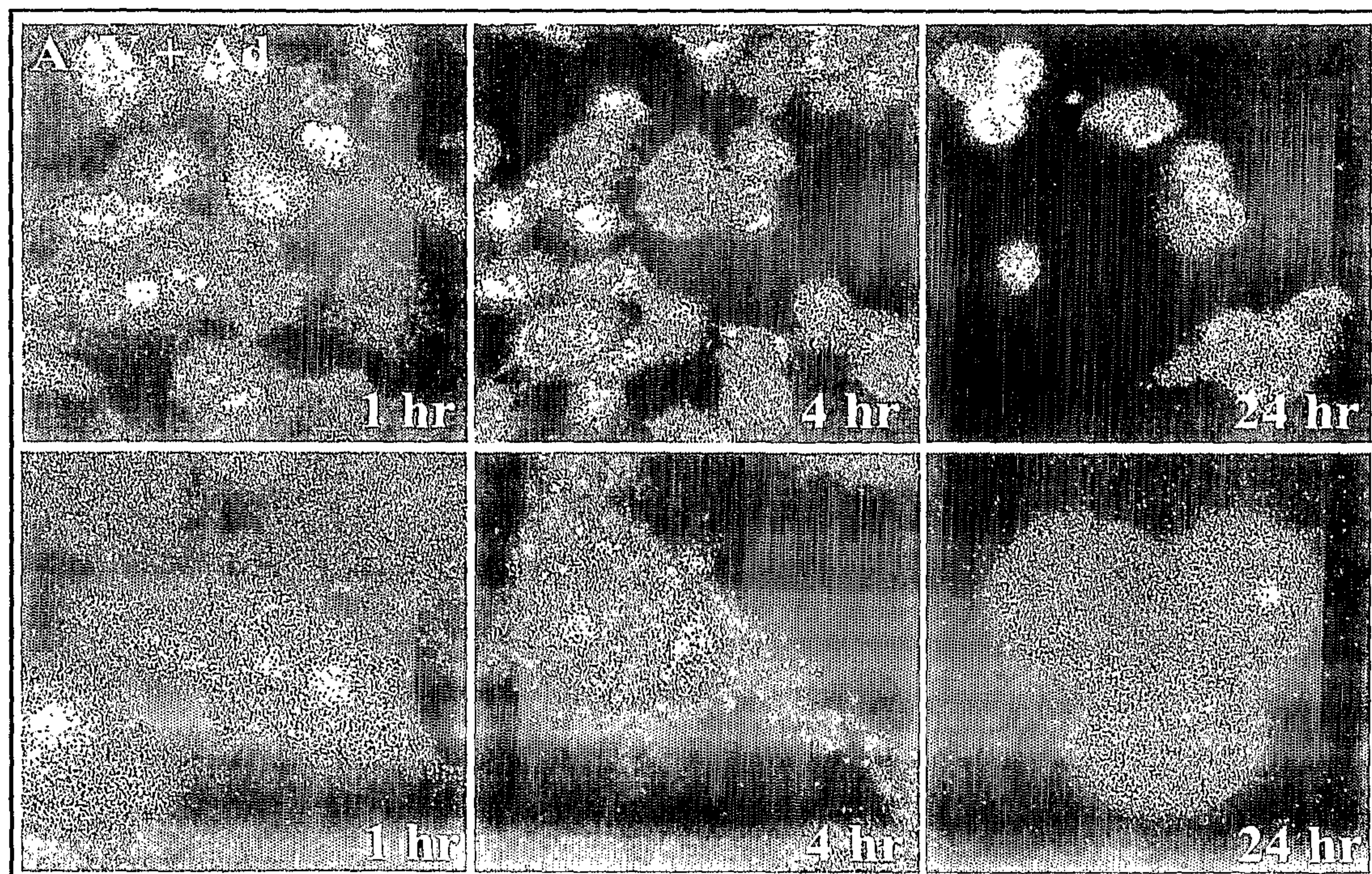


FIG. 31B