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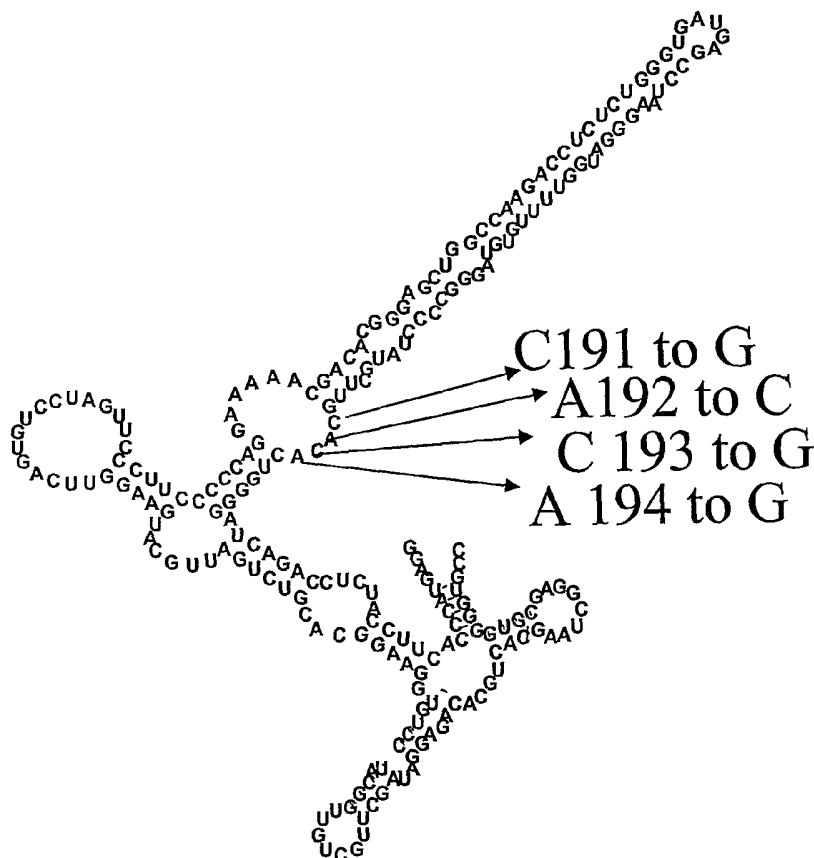
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[Continued on next page]

(54) Title: POTENT COMBINATIONS OF MRNA TRANSPORT ELEMENTS

(57) Abstract: This invention provides expression vectors that comprise a combination of RNA transport elements that improve expression.



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## POTENT COMBINATIONS OF mRNA TRANSPORT ELEMENTS

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application nos. 60/471,988, filed May 19, 2003, and 60/472,223, filed May 20, 2003, each of which applications is herein  
5 incorporated by reference.

### BACKGROUND OF THE INVENTION

[0002] Efficient transport of the mRNA is an important step for achieving efficient expression. RNA transport is controlled by a complex interaction of cellular proteins with the mRNAs in the nucleus, leading to cytoplasmic export and expression. Two types of  
10 potent retroviral RNA transport elements have been identified. The constitutive transport element (CTE) is essential for the nucleo-cytoplasmic export of unspliced RNA of the simian type D retroviruses. (Bray, *et al.*, *Proc Natl Acad Sci USA* 91:1256-1260, 1994; Tabernero, *et al.*, *J Virol* 71:95-101, 1997; Tabernero, *et al.*, *J Virol* 70:5998-6011, 1996). A related element was identified in a subclass of rodent intracisternal A particle retroelements  
15 (Tabernero, *et al.*, *J Virol* 71:95-101, 1997). The RNA transport element (RTE) is present in another subset of rodent intracisternal A particle retroelements (Nappi, *et al.*, *J Virol* 75:4558-69 2001). The primary sequence of these elements are distinct. It has been demonstrated that both CTE and RTE are posttranscriptional control elements promoting expression of otherwise poorly expressed mRNAs such as gag and env from a mutant HIV  
20 lacking the viral Rev-RRE control mechanism(Nappi, *et al.*, *J Virol* 75:4558-69 2001; Valentin, *et al.*, *J Virol* 71:9817-9822, 1997; von Gegerfelt & Felber. *Virology* 232:291-299, 1997)

[0003] CTEs and RTEs are RNA binding sites for distinct cellular factors mediating the export of CTE- and RTE-containing mRNAs. RTEs and CTEs fold into distinct extended  
25 RNA stem-loop structures. Both CTEs and RTEs function in cells of different species, indicating conserved cellular export mechanisms. While the cellular TAP/NXF1 has been identified to be the CTE export factor (Grüter, *et al.*, *Mol Cell* 1:649-659, 1998) the cellular factor promoting RTE export is still unknown.. TAP/NXF1 does not bind RTE (Nappi, *et al.*, *J Virol* 75:4558-69 2001). However, using a microinjected *Xenopus* oocyte RNA transport  
30 assay, it was found that CTE RNA can compete for RTE export, indicating, that TAP/NXF1

is involved in RTE RNA export. Not to be bound by theory, the current working model implies another factor interacting with RTE and acting as adapter to link RTE to the export receptor TAP/NXF1.

5 [0004] As noted above, an important step in achieving efficient expression in eukaryots is RNA transport. The role of CTEs and RTEs in RNA transport has been studied individually. The presence of more than one copy of CTE in some, but not all, cases has shown increases in expression relative to one copy of CTE alone (Wodrich *et al Nucl Acid Res* 28:901-910, 2000). However, the effects of the combination of CTE and RTE have not been evaluated.

10 [0005] This invention provides a novel tool to improve expression of unstable mRNAs. The invention provides a modified RTE element that functions better than the wildtype element. Moreover, the invention provides vectors comprising a combination of an RTE, *e.g.*, the mutant RTE, and a CTE, which combination enhances gene expression. Since the function of these elements is conserved in mammalian cells, the use of the invention (RTE-CTE combination of RNA export elements) provides a simple method to improve gene  
15 expression to levels otherwise only achieved via more cumbersome RNA optimization.

[0006] In addition, the combination RTE/CTE has an added advantage in that it does not duplicate sequences, as is the case of vectors having multiple CTEs. This can be important, as the vectors are typically grown in bacteria and sequence duplication is often not well tolerated in many bacterial systems.

20 [0007] This invention thus provides vectors and methods of using the vectors to obtain high level expression of genes, including for applications such as gene therapy. Further, the RTE/CTE combination can be used in any gene transfer scenario where genes may be poorly expressed, *e.g.*, DNA-based cytokine or viral antigen vaccine vectors. This applies to the field of retroviral vector development, the development of DNA-based vaccine vectors and  
25 other applications to enhance gene expression.

#### BRIEF SUMMARY OF THE INVENTION

[0008] The invention provides two RNA transport elements that in combination enhance expression of a transcript that comprises the two transport elements. These elements are the constitutive transport element (CTE), which is important for the nucleo-cytoplasmic export of  
30 the unspliced RNA of the simian type D retroviruses. The other element is the RNA

transport element, RTE, which is present in a subset of rodent intracisternal A particle retroelements.

[0009] In one aspect, the invention provides a vector, *e.g.*, a lentiviral vector, retroviral vector, adenoviral vector, adeno-associated viral vector, or plasmid, comprising an expression cassette encoding a transcript that comprises a RTE and a CTE, wherein the presence of the RTE and CTE increases expression of the transcript. In typical embodiments, the RTE is an RTE<sub>M26</sub>. For example, the RTE<sub>M26</sub> can comprise the sequence set forth in SEQ ID NO:2. In one embodiment, the RTE<sub>M26</sub> and the CTE are positioned in the 3' untranslated region of a transcript encoded by the expression cassette. Often, the RTE and the CTE are separated by 100 nucleotides or less. In some embodiments, the RTE and the CTE are separated by 30 nucleotides or less. In one embodiment, the RTE and the CTE are comprised by the sequence set forth in SEQ ID NO:3. The expression cassette can comprise a sequence encoding a viral gene product, for example, a retroviral gene product such as an HIV-1 gag or HIV-1 env.

[0010] In another aspect, the invention provides a vector comprising an expression cassette encoding a transcript that comprises an RTE<sub>M26</sub>.

[0011] The invention also provides methods of using the vectors to enhance expression of a gene product of interest.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 shows the modeled structure and sequence of RTE<sub>M26</sub>.

[0013] Figure 2 shows a comparison of wildtype RTE and a mutant RTE (RTE<sub>M26</sub>).

[0014] Figure 3 shows the gag reporter plasmid and the sites where the RTE and CTE are inserted.

[0015] Figure 4 provides exemplary data showing that RTE<sub>M26</sub> increases gag expression in comparison to RTE. Values are shown with standard deviations.

[0016] Figure 5 shows the sequence of RTE<sub>M26</sub>-CTE.

[0017] Figure 6 provides exemplary data that shows that RTE<sub>M26</sub>CTE increases gag expression.

[0018] Figure 7 provides exemplary data that shows that enhanced expression mediated by RTE-CTE combinations is independent of cell type. The dark shading represents

experiments performed in HeLa HLtat cells. The lighter shading represents experiments performed in human 293 cells.

[0019] Figure 8 provides exemplary data showing that enhanced expression mediated by RTE-CTE combinations is independent of splice sites.

5 [0020] Figure 9 shows the effects of spacing on the enhanced expression mediated by RTE26/CTE combinations.

[0021] Figures 10A and 10B present exemplary data that show the effect of RTE26-CTE on HIV-1 envelope expression. Figure 10 A provides a schematic of the expression vector. The env cDNA is expressed from HIV-1 LTR promoter (Schwartz *et al J. Virol.* 64: 5448-  
10 5456, 1990) Figure 10B shows the results of a Western blot analysis of the levels of expressed env protein. The lanes show the level of expressed env protein from env cDNA clone pNL1.5E containing the indicated RNA export elements. The results show that RTE26-CTE mediates enhanced expression of HIV-1 env.

[0022] Figures 11A and 11B provides exemplary data showing that RTE26CTE  
15 synergistically increases expression from molecular clone SIV R-R- $\Delta$ nef. Figure 11A provides a schematic of the Rev(-)RRE(-) $\Delta$ nefCTE(+)-SIVmac239 clone (Von Gegerfelt *et al. Virology* 232:291-299, 1997; *J. Virol.* 73:6159-6165, 1999; *J. Virol.* 76:96-104, 2002) into which RTE26 was cloned. Figure 11A shows the generation of Rev-independent molecular clones of SIVmac239 containing the RTE26CTE RNA export element. Figure  
20 11B provides data showing the expression of the plasmid in 293 cells. The results show that RTE26CTE mediates increased expression from molecular clone SIV R-R- $\Delta$ nef

[0023] Figure 12 provides exemplary data showing that the position of the individual elements within the RTE/CTE combination is interchangeable.

[0024] Figure 13 provides exemplary data showing that the length of the spacer between  
25 the RTE and CTE elements is important for optimal function.

[0025] Figure 14 provides a schematic of the structure of RTE showing important structural features.

[0026] Figure 15A shows a structural prototype of each of four major RTE families that were identified based RTE-like elements in the mouse genome. Conservation of overall

structural arrangement is shown. Figure 15B shows the alignment of the four prototype family members. Figure 15C shows the alignment of the consensus of each family.

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

5 [0027] The term "RTE" refers to a post-transcriptional regulatory element that is a *cis*-acting nucleic acid sequences involved in the transport, stability, and translation of RNA transcripts. Exemplary RTE sequences are provided in WO 99/61596. RTE folds into an extended RNA stem-loop structure. RTE sequences for use in the invention have at least 60% identity, often 70%, 80%, 85%, 90%, 95%, or greater identity, as determined with  
10 reference to the cores sequence shown in SEQ ID NO:1.

[0028] An "RTEM26" as used herein refers to an RTE that comprises four nucleotide substitutions at the positions indicated in Figure 1. An RTEM26 has at least 60% identity, often 70%, 80%, 85%, 90%, 95%, or greater identity, as determined with reference to the core sequence shown in SEQ ID NO:2.

15 [0029] The term "CTE" refers to a *cis*-acting constitutive transport element that functions in RNA transport. Exemplary CTE sequences are provided, *e.g.*, in Tabernero, *et al.*, *J. Virol.* 70:5998-6011, 1996; and US Patent Nos. 5,880,276 and 5,585,263. CTE sequences for use in the invention have at least 60% identity, often 70%, 80%, 85%, 90%, 95%, or greater identity, as determined with reference to the exemplary sequence SEQ ID NO:4.

20 [0030] "Enhanced expression" in the context of this invention refers to increased expression relative to a control. An increase in expression is achieved when the value relative to the control is 110%, more preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

[0031] The term "HIV" refers to a lentivirus usually called "human immunodeficiency virus" which is believed to be the causal agent of acquired immune deficiency syndrome, or  
25 AIDS. There are several known subtypes of HIV, including HIV-1 and HIV-2. HIV and AIDS are well described in the literature and, *e.g.*, are further described by Gottfredsson (1997) *Front Biosci.* 2: D619-D634; Burton (1997) *Proc. Natl. Acad. Sci. USA* 94:10018-10023; Barnadas (1997) *J. Cutan. Pathol.* 24:507-510; Doms (1997) *Virology*  
30 235:179-190; Cossarizza (1997) *AIDS* 11:1075-1088; Carpenter (1997) *JAMA* 277:1962-1969; Klein (1995) *Trends Microbiol.* 3:386-391.

[0032] The term "wild-type" refers to any form (*e.g.*, tertiary structure), structure (*e.g.*, secondary structure) or sequence (*e.g.*, primary structure) of a composition, *e.g.*, a nucleic acid, as found in nature, versus structures or sequences that have been manipulated by man.

[0033] An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[0034] The term "expression cassette" refers to any recombinant expression system for the purpose of expressing a nucleic acid sequence of the invention *in vitro* or *in vivo*, constitutively or inducibly, in any cell, including prokaryotic, yeast, fungal, plant, insect or mammalian cell. The term includes linear or circular expression systems. The term includes expression systems that remain episomal or integrate into the host cell genome. The expression systems can have the ability to self-replicate or not, *i.e.*, drive only transient expression in a cell. The term includes recombinant expression cassettes which contain only the minimum elements needed for transcription of the recombinant nucleic acid. The term also includes expression vectors. The mutant RTE and RTE/CTE combination sequences can be incorporated into any eukaryotic expression system, typically mammalian expression systems, to improve expression of unstable RNAs.

[0035] The term "isolated," when referring to a molecule or composition, such as, for example, a polypeptide or nucleic acid, means that the molecule or composition is separated from at least one other compound, such as a protein, other nucleic acids (*e.g.*, RNAs), or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a polypeptide or nucleic acid is considered isolated when it has been isolated from any other component with which it is naturally associated, *e.g.*, cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state and can be in a dry or an aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemistry techniques such as polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC).

[0036] The term "polynucleotide," "nucleic acid molecule" or "nucleic acid sequence" refers to a deoxyribonucleotide or ribonucleotide oligonucleotide in either single- or double-



stranded form. The term encompasses nucleic acids, *i.e.*, oligonucleotides, containing known analogues of natural nucleotides which have similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also includes nucleic acids which are metabolized in a manner similar to naturally occurring nucleotides or at rates that are improved thereover for the purposes desired. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs, which contain non-ionic backbones, such as N-(2-aminoethyl) glycine units); see *Oligonucleotides and Analogues, A Practical Approach*, edited by F. Eckstein, IRL Press at Oxford University Press (1991); *Antisense Strategies*, *Annals of the New York Academy of Sciences*, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) *J. Med. Chem.* 36:1923-1937; *Antisense Research and Applications* (1993, CRC Press) in its entirety and specifically Chapter 15, by Sanghvi. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) *Toxicol Appl Pharmacol* 144:189-197. Other synthetic backbones encompassed by the term include methylphosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) *Biochemistry* 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) *Antisense Nucleic Acid Drug Dev.* 6:153-156). The term nucleic acid is used interchangeably with gene, DNA, cDNA, RNA, mRNA, oligonucleotide primer, probe and amplification product.

[0037] The term "exogenous" as in "exogenous nucleic acid" refers to a molecule (*e.g.*, nucleic acid or polypeptide) that has been isolated, synthesized, and/or cloned, in a manner that is not found in nature, and/or introduced into and/or expressed in a cell or cellular environment other than or at levels or forms different than the cell or cellular environment in which said nucleic acid or protein can be found in nature. The term encompasses both nucleic acids originally obtained from a different organism or cell type than the cell type in which it is expressed, and also nucleic acids that are obtained from the same organism, cell, or cell line as the cell or organism in which it is expressed.

[0038] The term "endogenous" refers to a molecule, *e.g.*, a nucleic acid or polypeptide, in a form, structure and/or sequence found in nature.

[0039] "Sequence identity" in the context of two nucleic acid sequences includes reference to the nucleotides in the two sequence sthat are the same when aligned for maximum correspondence over a specified "comparison window." Sequence identity analysis is used to determine whether a nucleic acid is within scope of the invention. "Sequence identity" can be analyzed by optimal alignment of sequences for comparison using any means to analyze sequence identity (homology) known in the art, *e.g.*, by the progressive alignment method of termed "PILEUP"; by the local homology algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2: 482; by the homology alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443; by the search for similarity method of Pearson (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444; by computerized implementations of these algorithms, *e.g.* BLAST, GAP, BESTFIT, FASTA, and TFASTA in, *e.g.*, the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI; or, by inspection. See also Morrison (1997) *Mol. Biol. Evol.* 14:428-441, as an example of the use of PileUp, ClustalW, TreeAlign, MALIGN, and SAM sequence alignment computer programs. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle (1987) *J. Mol. Evol.* 35:351-360, and is similar to the method described by Higgins & Sharp (1989) *CABIOS* 5:151-153. The BLAST algorithm is described in Altschul (1990) *J. Mol. Biol.* 215: 403-410, and BLAST software for analyses is publicly available, *e.g.*, see National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/>. See also Corpet (1988) *Nucleic Acids Res.* 16:10881-90; Huang (1992) *Computer Applications in the Biosciences* 8:155-65; Pearson (1994) *Methods in Molec. Biol.* 24:307-31. For purposes of this application, the percent identity can be determined using the default parameters of BLAST 2.0 without the filter. "Determined with reference" in this context means that the sequences are determined when aligned for maximal correspondence over the reference sequence.

[0040] The term "recombinant," when used with reference to, *e.g.*, a cell, nucleic acid, polypeptide, expression cassette or vector, refers to a material, or a material corresponding to the natural or native form of the material, that has been modified by the introduction of a new moiety or alteration of an existing moiety, or is identical thereto but produced or derived from synthetic materials. For example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell (*i.e.*, "exogenous nucleic acids") or express native genes that are otherwise expressed at a different level, typically, under-expressed or not expressed at all. The term "recombinant means" refers to techniques where, *e.g.*, a recombinant nucleic acid such as a cDNA encoding a protein or an antisense sequence, is

inserted into an expression cassette, such as an expression vector, the resultant construct is introduced into a cell, and the cell expresses the nucleic acid, and the protein, if appropriate. "Recombinant means" also encompass the ligation of nucleic acids to coding or promoter sequences from different sources into one expression cassette or vector for expression of a fusion protein, constitutive expression of a protein, or inducible expression of a protein.

[0041] The term "motif" or "domain" refers to a nucleic acid sequence pattern, or structure, which is shared between related molecules.

[0042] The term "ameliorating" or "ameliorate" refers to any indicia of success in the treatment of a pathology or condition, including any objective or subjective parameter such as abatement, remission or diminishing of symptoms or an improvement in a patient's physical or mental well-being. Amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination and/or a psychiatric evaluation.

[0043] The term "vaccine" is used in its ordinary sense, meaning an agent which is capable of eliciting a humoral and/or cell-mediated immunoprotective immune response when administered to an individual with an at least partially functioning immune system.

[0044] The term "prophylaxis" refers to any form of prevention, delay or abatement a pathology or condition or symptom thereof, including any objective or subjective parameter.

### Detailed Description

[0045] This invention relies on routine techniques in the field of recombinant genetics.

Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994-April 2004 update).

[0046] The invention provides for methods and reagents for using the RTE-CTE combination in various expression vectors to improve expression levels in cells, *e.g.*, in eukaryotic cells such as mammalian cells. The construction of such expression vectors are well known in the art. Expression constructs contain various elements to mediate expression of a transcript encoded by a gene of interest. These elements include regulatory elements, *e.g.*, promoters and/or enhancers linked to the sequences to be expressed. Such sequences are well known and include both constitutive and inducible or tissue-specific promoters.

[0047] Expression constructs typically contain at least one independent terminator sequence, other sequences to optimize expression, *e.g.*, IRES, splice donor and splice acceptor sites, polyadenylation sequences, sequences permitting replication of the cassette in eukaryotes, or prokaryotes, or both, (*e.g.*, shuttle vectors) and selection markers for both prokaryotic and eukaryotic systems. These components are also well known. Further, kits for many such expression systems are commercially available.

#### RTE sequences

[0048] RTE sequences that are used in the invention include naturally occurring RTEs and variants that retain their function. RTE sequences can be determined with reference to either the wildtype RTE sequence or RTE<sub>M226</sub> sequence shown in Figures 1 and 2. The RTE contains four stem-loops that are typically required for function in mammalian cells. Only part of the element is needed to mediate RNA transport in non-mammalian cells, *e.g.*, *Xenopus*. RTE sequences are known in the art, *e.g.*, WO 99/61596 and Nappi, *et al.*, *J Virol* 75:4558-69 2001.)

[0049] The RTEs of the invention often comprise mutations at any one of the 4 positions indicated in Figures 1 and 2 (positions 191, 192, 193, and 194). These substitutions may be the particular substitutions shown in Figure 2, or may be substitutions with nucleotide or nucleotide analogs that result in the same activity. In some embodiments, the RTE comprises at least two or at least three of the mutations shown in Figure 2. In a preferred embodiment, a variant RTE that comprises all four of the mutations shown in the RTE<sub>M226</sub> sequence of Figure 1 and Figure 2 is used in the invention.

[0050] The invention includes the use of variant RTE sequences that differ from the wildtype RTE at positions outside of the 4 positions shown in Figures 1 and 2. Such variant sequences maintain the stem-loop structure and typically have at least 200 contiguous nucleotides of the wt RTE and RTE<sub>M26</sub> sequence shown in Figure 2. For example, variants can comprise nucleotide substitutions at positions that participate in a nucleotide-nucleotide binding interaction, *e.g.*, positions shown in Figure 1. In such cases, the variant also typically comprises a compensatory substitution such that the structure of the RTE is retained.

[0051] A schematic of the complex structural arrangement of RTE is shown in Figure 14. As in the case of CTE, several structural regions within the RTE are essential in terms of function, but not the primary sequence. These regions are not the primary interaction site for the cellular factor mediating export. Thus, substitutions may be present in these regions

relative to the sequence shown in Figure 14, so long as structure is conserved. With regard to Figure 14, SL I and SL II are dispensable for transport function from *Xenopus* oocyte nucleus, but are important for mRNA export from mammalian cells.

5 [0052] A search of the mouse genome identified more than 3000 RTE-like elements in that fall into 4 major families. A prototype of each family is shown in Figure 15 A (structure) and Figure 15B (alignment). Figure 15C shows the alignment of the consensus of each family. There is a conservation overall structure. One difference among them is stem loop IV, but experimentally, either loop functions. Thus, RTEs for use in the invention can also comprise variants in stem loop IV as well as other variants that maintain the overall structure.

10 [0053] RTE variants can be evaluated for function by the ability to mediate RNA transport using known assays, *e.g.*, using an assay such as a *Xenopus* oocyte assay. Exemplary functional assays are provided in the examples and in WO 99/61596. RTEs for use in the invention increase expression at a level equivalent to that of wt RTE or RTE<sub>M26</sub>.

#### CTE sequence

15 [0054] CTE sequences are known in the art. CTE sequences for use in the invention include variant CTEs that retain the ability to mediate RNA export. Exemplary CTE sequences are provided, *e.g.*, in von Gegerfelt & Felber, *Virology* 232:291-299, 1997; and U.S. Patent No. 5,880,276; Bray, *et al.*, *Proc Natl Acad Sci USA* 91:1256-1260, 1994; and Tabernero, *et al.*, *J Virol* 70:5998-6011, 1996.

20 [0055] In simian retrovirus type 1 (SRV-1), the core CTE spans 173 nucleotides and is sufficient for the propagation of a RRE/Rev-deficient molecular clone of HIV-1. Computer analysis and confirming mutagenesis studies suggest an RNA secondary structure consisting of an extended stem-bulge-loop. Similar structures are obtained for the simian retrovirus type 2 (SRV-2) and Mason-Pfizer monkey virus (MPMV) CTEs. SRV-1, SRV-2, and MPMV  
25 have high degrees of sequence homology in the regions panning the core CTE: 88% between SRV-1 and SRV-2 and 92% between SRV-1 and MPMV.

[0056] Variant CTEs for use in this invention typically maintain the stem loop structure, including maintaining the distance between the two loops. (See, *e.g.*, Tabernero, *et al.*, *J Virol* 70:5998-6011, 1996, for a schematic of the SRV-1CTE). Exemplary variants that  
30 maintain function are often in regions that are not conserved between the SRV-1, SRV-2, and MPMV sequences. For example, nucleotide composition of the hairpin is not absolutely

conserved between SRV-2, MPMV, and SRV-1. Tabernero *et al, supra*, provide additional guidance in generating variant CTE sequences. For example, mutant 19 (Tabernero, *et al.*, *J Virol* 70:5998-6011, 1996 on page 6005) has 12 of 173 nts that are different relative to the SRV-1 sequence, but maintains the stem structure separating the internal loops. This mutant functions like wild type CTE, while mutant 17, in which this region is deleted, is inactive. Thus, variants useful in the invention include those that have compensatory changes, but do not affect the two internal loop regions that represent the binding sites for the cellular export factor TAP/NXF1.

[0057] Exemplary assays for evaluating CTE function are provided in the examples and in reference cited herein. CTEs for use in the invention have a level of function that is equivalent to a wildtype CTE.

#### Positioning of the RTE and CTE elements

[0058] The vectors of the invention comprise expression cassettes that encode transcripts having both an RTE and CTE present. The RTE and CTE may be in any order, *i.e.*, the RTE may be present upstream of the CTE; or the CTE may be present upstream of the RTE. The spacing between the RTE and CTE is typically less than 200, 100, or 50 nucleotides. In some embodiments, the RTE and CTE are separated by 30 nucleotides or less.

[0059] The RTE and CTE combination can be present in any position, *e.g.*, 5' to the gene to be expressed, 3' to the gene to be expressed, or within an intron, but is typically contained in the 3' untranslated region of a transcript.

#### Determination of increased expression

[0060] The ability of an RTE/CTE combination to enhance expression can be determined using any of a number of assays that are well-known in the art. Enhanced expression can be determined relative to a control that comprises only an RTE, *e.g.*, shown in Figure 1, or only a CTE, *e.g.*, SEQ ID NO:4.

[0061] The level of expression of a gene can be analyzed by measuring the level of RNA transcript and/or by measuring the level of a protein encoded by the gene. RNA transcript levels can be determined using various techniques, including reverse transcription and amplification of, northern blotting, dot blotting, RNase protection, and the like. The level of a protein can be determined using convenient techniques such as western blotting or other

immunoassays that employ an antibody to the protein encoded by the gene of interest. Alternatively, protein activity levels could also be used to assess gene expression.

[0062] Any gene of interest can be encoded by the expression vectors of the invention. For example, it may be desirable to use the RTE/CTE combination to enhance expression of genes that can be difficult to expression, *e.g.*, lymphokines or cytokines. Examples of cytokines include GM-CSF, the interleukins, especially IL-1, IL-2, IL-4, IL-12, IL-10, IL-19, IL-20, interferons of the alpha, beta and gamma subtypes. Chemokines can also be delivered using expression vectors of the invention. Examples of chemokines include platelet factor 4 (PF4), platelet basic protein (PBP), interleukin-8 (IL-8), melanoma growth stimulatory activity protein (MGSA), macrophage inflammatory protein 2 (MIP-2), pre-B cell growth stimulating factor (PBSF), monocyte chemotactic proteins, macrophage inflammatory proteins, RANTES, and many others.

[0063] Other genes of interest can also be delivered for therapeutic applications. Examples of genes for therapeutic application include genes that encode immunogenic proteins for which it is desired to induce a cellular and/or humoral immune response and genes that encode cytostatic products where the expression of the product in a cell produces an arrest in the cell cycle (*e.g.*, p21, the retinoblastoma gene product, and the like). The expression vectors of the invention can also be used to deliver growth factors or other hormones, *e.g.*, insulin, growth hormone, nerve growth factor, vascular endothelial growth factor, and various polypeptides that have angiogenic or anti-angiogenic activity; gene products involved in apoptosis or tumor suppression; or pro-drug activating genes.

[0064] Further an RTE/CTE combination can be used to enhance transcript levels of other sequences, *e.g.*, RNA sequences, encoded by an expression vector. These include sequences such as antisense RNA sequences or ribozymes.

#### Introduction of expression vectors into cells

[0065] Standard transfection methods are used to introduce the expression vectors of the invention into cells. The expression vectors can be plasmid expression vectors or other commonly used expression vectors such as viral expression vectors. Gene transfer techniques include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing heterologous nucleic acids into a host cell (*see, e.g.*, Russell & Sambrook, *supra*). The vectors can be used for *in vitro* experiments or *in vivo*.

[0066] The cells are typically mammalian cells, *e.g.*, human cells. Cells into which the vectors are introduced can be primary cells as well as cell lines. Exemplary cell types include circulating cells such as peripheral blood cells, monocytes, lymphocytes, and cells of these lineages, including CD4<sup>+</sup> T cells, and the like; muscle cells, epidermal cells, neuronal cell types, fibroblasts, hepatocytes, cardiac cells, mammary cells, prostate cells, pancreatic cells, lung cells, endocrine cells, splenocytes, and the like. Such cells may be normal or cancerous.

*Non-viral delivery methods*

[0067] Methods of non-viral delivery of nucleic acids comprising the RTE/CTE combinations of the invention include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in *e.g.*, US 5,049,386, US 4,946,787; and US 4,897,355) and lipofection reagents are sold commercially (*e.g.*, Transfectam<sup>TM</sup> and Lipofectin<sup>TM</sup>). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells either *in vitro* or *in vivo*.

[0068] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

*Viral delivery methods*

[0069] The use of RNA or DNA viral based systems for the delivery of vectors comprising the RTE/CTE combinations are known in the art. Conventional viral based systems can include lentivirus, retroviral, adenoviral, adeno-associated, herpes simplex virus, and various other viral vectors for gene transfer.

[0070] In many applications, it is desirable a vector be delivered with a high degree of specificity to a cell type, *e.g.*, for delivery *in vivo*. A viral vector can be modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat



protein on the viruses outer surface. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *PNAS* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other pairs of virus expressing a ligand fusion protein and target cell expressing a receptor. For example, filamentous phage can be engineered to display antibody fragments (*e.g.*, Fab or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences thought to favor uptake by specific target cells.

[0071] Vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can also be delivered to cells *in vitro*. Such methods include *ex vivo* methods, *e.g.*, for introducing DNA into cells explanted from an individual patient.

[0072] *Ex vivo* cell transfection for diagnostics, research, or for gene therapy (*e.g.*, via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In some embodiments, cells are isolated from the subject organism, transfected with expression vectors comprising the RTE/CTE combination and re-infused back into the subject organism (*e.g.*, patient). Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (see, *e.g.*, Freshney *et al.*, *Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

[0073] Vectors (*e.g.*, lentiviruses, retroviruses, adenoviruses, liposomes, etc.) containing therapeutic nucleic acids can also be administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[0074] The data obtained from cell culture assays and animal studies can be used to formulate a dosage range for use in humans. The dosage can vary within this range depending upon the dosage form employed and the route of administration.

[0075] When administering a viral vector, the amount of virus (number of virions) per dose will vary depending on results of different titrations used in clinical trials. The range can range, *e.g.*, from only a few infectious units, to about  $10^4$  to  $10^{10}$  infectious units (*i.e.*, virions) per dose. Protocols and means to determine safety and efficacy used for other attenuated vaccines can be adapted and used with the novel reagents provided by the invention; see, *e.g.*, Belshe (1998) *N. Engl. J. Med.* 338:1405-1412; Gruber (1997) *Vaccine* 15:1379-1384; Tingle (1997) *Lancet* 349:1277-1281; Varis (1996) *J. Infect. Dis.* 174:S330-S334; Gruber (1996) *J. Infect. Dis.* 173:1313-1319.

[0076] The vaccine can be administered in conjunction with other treatment regimens, *e.g.*, it can be coadministered or administered before or after any anti-viral pharmaceutical (see, *e.g.*, Moyle (1998) *Drugs* 55:383-404) or a killed (completely inactivated) anti-HIV vaccine. The vaccine can be administered in any form of schedule regimen, *e.g.*, in a single dose, or, using several doses (*e.g.*, boosters) at dosages and time intervals to be determined by clinical trials.

[0077] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention, as described below (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed., 1989).

## Kits

[0078] The invention also includes kits that contain the RTE/CTE combination expression vectors. Such kits can be prepared from readily available materials and reagents.

## EXAMPLES

### Example 1. The presence of REM and CTE in combination enhances expression

[0079] Several mutant RTE elements were tested. Of these RTE26 (Fig. 1 to 4) increased gag expression by 2-fold as compared to the wild type element (Fig. 4). The RTE

element RTEM26 has 4 nucleotides changed, which does not affect the modeled structure shown in (Figure 1).

[0080] We also tested several mutants of CTE to mediate expression of HIV-1 gag in mammalian cells. These mutants did not function better than the wild-type CTE from simian type D retrovirus-1 (SRV-1) (*see, e.g.,* Tabernero, *et al., J Virol* 71:95-101, 1997; Tabernero, *et al., J Virol* 70:5998-6011, 1996).

[0081] We found that a combination of a mutated RTE (RTEM26) positioned next to the CTE in *cis* (Fig 5) has a potent effect on mRNA transport, leading to a more than 10x improved expression of unstable mRNAs, compared to either element alone. Using the HIV-1 gag as reporter mRNA, we found that RTE alone and CTE alone result in ~20x and ~50x increased expression, respectively, while the combination of these elements yielded about a ~500x fold increase in Gag expression (Fig 6). Similar levels of green fluorescent protein (GFP) were produced from co-transfected plasmid pFRED25, used as the internal control. Therefore, the combination of RTEM26 and CTE provides a unique method to greatly activate expression of gag, which is otherwise expressed very poorly.

[0082] We further showed that this effect is independent of the cell type, since similar data were obtained in HeLa and 293 cells (Fig 7).

[0083] We also found that this effect on the gag mRNA is independent of splice sites, since we inserted RTEM26, CTE and the combination into pNLcgag, which lacks the major splice donor of HIV-1 located 5' to the gag gene (Fig 8). We also found a potent effect on gag expression from this plasmid. The ability of RTEM26-CTE to increase expression was influenced by the spacing. When RTEM26 and CTE were separated by 325 nucleotides, synergism was not observed. When RTEM26 and CTE were separated by a short distance, *e.g.,* 27 nt, enhanced expression was pronounced. Mutations in RTEM26 also markedly transduced an increase in expression relative to wildtype RTE.

## Materials and Methods

### *Generation of RTEM26*

[0084] The wild type RTE element spanning 226 nt (previously referred to as RTE 391-616 in Nappi, *et al., J Virol* 75: 4558-69, 2001 and now numbered 1-226) was used to introduce nucleotide changes using PCR methodology. The RTE designated RTEM26 has nucleotides (#190-193) CACA changed to GCGG.

*Description of NLgagRTE, NLgagRTEM26, NLgagCTE, NLgagRTEM26CTE*

[0085] We used the pNLgag expressing the HIV-1 p55gag gene as reporter plasmid (Felber, *et al.*, *Proc Natl Acad Sci USA* 86:1495-1499, 1989; Hadzopoulou-Cladaras, *et al.*, *J Virol* 63:1265-1274, 1989; Solomin, *et al.*, *J Virol* 64:6010-6017, 1990). In this plasmid, the gag gene is flanked by the HIV-1 LTRs which act as promoter and polyadenylation signal, respectively (Fig 3). The RTE and RTEM26 were inserted into the SacII site located 3' to the gag gene. Subsequently, CTE was inserted into the XbaI-SalI sites located 3' to the RTEM26, generating the NLgagRTEM26CTE.

*transfection*

[0086] Human HLtat, a HeLa derivative producing HIV tat (used to activate the LTR promoter) or human 293 cells were transfected with 1 µg of the NLgag plasmids (for 293 cells a tat expression plasmid pBstat was cotransfected). We routinely analyzed 2-3 independent clones in duplicate. We contranfect pFRED25 (0.8 µg) as internal control. All transfections were performed using FUGENE-6. Two days later, the cells were harvested in Tris-Triton lysis buffer and the cell extracts were analyzed for Gag expression using a commercial (Zeptomatrix) HIV gag antigen capture assay and for GFP production.

Example 2. Enhanced expression of HIV-1 env.

[0087] An expression vector was constructed to express the HIV env protein (Figures 10A and 10B.) A schematic of the plasmid is shown in Figure 10A. To evaluate expression, 293 cells were transfected with the plasmid and 2 days later, cell extracts were prepared. The extracts were analyzed for HIV env expression by Western blot using anti-HIV env serum. The levels of the env produced from the cDNA containing the RRE was very low in the absence of Rev, while the presence of Rev led to a notable increase (undiluted, 1:10, and 1:100 dilute samples are shown in Figure 10B). Both RTE and CTE mediated an increase in env production, but the level were about 10% of those obtained in the presence of Rev. The presence of RTEM26CTE led to levels comparable to those obtained in the presence of Rev (undiluted, 1:10, and 1:100 dilutions).

Example 3. RTEM26CTE mediate enhanced expression from the molecular clone SIV R-R-Δnef

[0088] Figure 11A shows a schematic of the Rev(-)RRE(-)Δnef CTE(+)SIVmac239 clones into which RTEM26 was cloned. Expression of the plasmid was evaluated in 293 cells.

Western blot analysis shows a dramatic increase in expression of the RTE/CTE-  
containing cells compared to cells transfected with a plasmid containing CTE only. The  
levels are similar to those obtained from the wild type virus. This shows that RTE/CTE  
is a powerful mediator of expression of a complex array of mRNAs produced from the full  
length mRNA.

[0089] The RTE/CTE combination was also tested in plasmids that were previously  
optimized for expression. The presence of RTE/CTE in such vectors elevated expression  
about 2-3-fold in human 293 cells (data not shown). In some embodiments, an RTE/CTE  
combination can be used in previously optimized vectors to introduce the vectors into  
primary cells for increased expression.

#### Example 4. RTE/CTE configuration

[0090] The configuration of the RTE/CTE combination was also examined. Figure 12  
provides exemplary data showing that the position of the individual elements within the  
RTE/CTE combination is interchangeable. The RTE/CTE in RTE/CTE is 5' to CTE,  
while in CTERTE/CTE it is 3' to the CTE.

[0091] Figure 13 shows that the length of the spacer between the element between 28 nt  
(original RTE/CTE) is important for optimal function. Increasing the distance to 202 or  
325 nt reduces the effect of RTE/CTE. Distances between the elements of 100 nt and 28  
nt yield similar results.

[0092] The above examples are provided by way of illustration only and not by way of  
limitation. Those of skill in the art will readily recognize a variety of noncritical parameters  
that could be changed or modified to yield essentially similar results.

[0093] All publications, patent applications, accession numbers, and other references cited  
in this specification are herein incorporated by reference as if each individual publication,  
patent application, accession number or other reference was specifically and individually  
indicated to be incorporated by reference.

**TABLE OF SEQUENCES****SEQ ID NO:1****RTE wildtype sequence**

5 CCGTGGGGTGCGAGGCTAAGCACTGCACAGAGGATAGCTTGCTGTTGGCATCCT  
 GTGGAAGGCACGTCTGATTGCATGAAGGTTTCAGTGTCTAGTTCCCTTCCCCCAG  
 GAAAAACGACACGGGAGCTGGCCAAGACCTCTCTGGGTGATGAGCCTAAGGGAT  
 GGTTTTGTGTAGGGCCCCTATGCTTGCACACTGGGGATCAGACCTCTACCTTCAC  
 CCATGAGG

10

**SEQ ID NO:2**

**RTEM26** nucleotide sequence of mutant RTEM26; the lower case letters indicate the four differences with RTEwt

15 CCGTGGGGTGCGAGGCTAAGCACTGCACAGAGGATAGCTTGCTGTTGGCATCCT  
 GTGGAAGGCACGTCTGATTGCATGAAGGTTTCAGTGTCTAGTTCCCTTCCCCCAG  
 GAAAAACGACACGGGAGCTGGCCAAGACCTCTCTGGGTGATGAGCCTAAGGGAT  
 GGTTTTGTGTAGGGCCCCTATGCTTGgcggCTGGGGATCAGACCTCTACCTTCACCC  
 ATGAGG

20 **SEQ ID NO:3**

**RTEM26CTE** nucleotide sequence of a preferred combination RTE-CTE element; lower case letters indicate the differences with the RTEwt and the spacer region.

25 CCGTGGGGTGCGAGGCTAAGCACTGCACAGAGGATAGCTTGCTGTTGGCATCCT  
 GTGGAAGGCACGTCTGATTGCATGAAGGTTTCAGTGTCTAGTTCCCTTCCCCCAG  
 GAAAAACGACACGGGAGCTGGCCAAGACCTCTCTGGGTGATGAGCCTAAGGGAT  
 GGTTTTGTGTAGGGCCCCTATGCTTGgcggCTGGGGATCAGACCTCTACCTTCACCC  
 ATGAGGtatcgataccgcgggtagctctagagtAGACCACCTCCCCTGCGAGCTAAGCTGGACA  
 GCCAATGACGGGTAAGAGAGTGACATTTTTCACTAACCTAAGACAGGAGGGCCG

TCAGAGCTACTGCCTAATCCAAAGACGGGTAAAAGTGATAAAAATGTATCACTC  
CAACCTAAGACAGGCGCAGCTTCCGAGGGATTG

**SEQ ID NO:4**

- 5 Exemplary CTE sequence. This CTE is from simian virus type I (SRV-1 (Tabernero *et al.*, *J. Virol.* 70:5998-6011, 1996).

AGACCACCTCCCCTGCGAGCTAAGCTGGACAGCCAATGACGGGTAAGAGAGTGA  
CATTTCCTACTAACCTAAGACAGGAGGGCCGTCAGAGCTACTGCCTAATCCAAAG  
ACGGGTAAAAGTGATAAAAATGTATCACTCCAACCTAAGACAGGCGCAGCTTCC  
10 GAGGGATTG

WHAT IS CLAIMED IS:

- 1                   1.       An expression vector comprising an expression cassette encoding a  
2 transcript that comprises a RTE and a CTE, wherein the presence of the RTE and CTE  
3 enhances expression of the transcript.
- 1                   2.       The vector of claim 1, wherein the RTE is an RTE<sub>M26</sub>
- 1                   3.       The vector of claim 2, wherein the RTE<sub>M26</sub> comprises the sequence  
2 set forth in SEQ ID NO:2.
- 1                   4.       The vector of claim 1, wherein the RTE<sub>M26</sub> and the CTE are  
2 positioned in the 3' untranslated region of a transcript encoded by the expression cassette.
- 1                   5.       The vector of claim 1, wherein the RTE and the CTE are separated by  
2 200 nucleotides or less.
- 1                   6.       The vector of claim 1, wherein the RTE and the CTE are separated by  
2 30 nucleotides or less.
- 1                   7.       The method of claim 6, wherein the RTE and the CTE are comprised  
2 by the sequence set forth in SEQ ID NO:3.
- 1                   8.       The vector of claim 1, wherein the expression cassette comprises a  
2 sequence encoding a viral gene product.
- 1                   9.       The vector of claim 8, wherein the sequence encoding a viral gene  
2 product is a retrovirus gene.
- 1                   10.      The vector of claim 9, wherein the retrovirus gene is HIV-1 *gag*.
- 1                   11.      The vector of claim 9, wherein the retrovirus gene is HIV-1 *env*.
- 1                   12.      The vector of claim 1, wherein the vector is a lentiviral vector
- 1                   13.      An expression vector comprising an expression cassette encoding a  
2 transcript that comprises an RTE<sub>M26</sub>.
- 1                   14.      A host cell comprising an expression vector of claim 1 or claim 14.



1                   15.    A method of enhancing expression of a transcript encoded by an  
2   expression vector, the method comprising introducing a vector comprising an expression  
3   cassette encoding a transcript that comprises a RTE and a CTE into a eukaryotic cell, wherein  
4   the presence of the RTE and CTE increases expression of the transcript.

1                   16.    The method of claim 15, wherein the RTE is an RTE<sub>M26</sub>

1                   17.    The method of claim 16, wherein the RTE<sub>M26</sub> comprises the sequence  
2   set forth in SEQ ID NO:2.

1                   18.    The method of claim 15, wherein the RTE<sub>M26</sub> and the CTE are  
2   positioned in the 3' untranslated region of a transcript encoded by the expression cassette.

1                   19.    The method of claim 15, wherein the RTE and the CTE are separated  
2   by 200 nucleotides or less.

1                   20.    The method of claim 15, wherein the RTE and the CTE are separated  
2   by 30 nucleotides or less.

1                   21.    The method of claim 20, wherein the RTE and the CTE are comprised  
2   by the sequence set forth in SEQ ID NO:3.

1                   22.    The method of claim 15, wherein the expression cassette comprises a  
2   sequence encoding a viral gene product.

1                   23.    The method of claim 22, wherein the sequence encoding a viral gene  
2   product is a retrovirus gene.

1                   24.    The method of claim 23, wherein the retrovirus gene is HIV-1 *gag*.

1                   25.    The method of claim 23, wherein the retrovirus gene is HIV-1 *env*.

1                   26.    The method of claim 15, wherein the vector is a lentiviral vector.

Figure 1

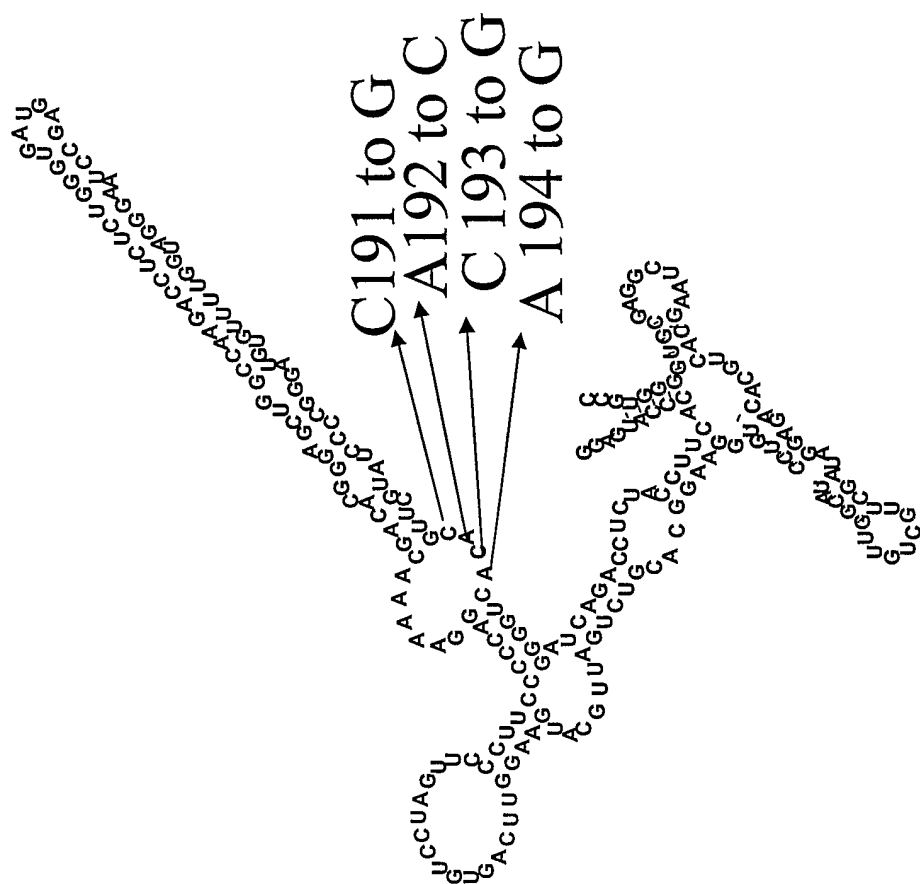


Figure 2

|       |   |     |                                                          |      |     |
|-------|---|-----|----------------------------------------------------------|------|-----|
| RTwt  | : | 1   | ccgtggggtcgaggctaagcacatgcacagaggatagcttgctgtggcatcctgt  | ggaa | 60  |
|       |   |     |                                                          |      |     |
| RTM26 | : | 11  | ccgtggggtcgaggctaagcacatgcacagaggatagcttgctgtggcatcctgt  | ggaa | 70  |
|       |   |     |                                                          |      |     |
| RTwt  | : | 61  | ggcacgtctgattgcatgaaggttcagtgctcctagttccctccccagggaaaaac | gaca | 120 |
|       |   |     |                                                          |      |     |
| RTM26 | : | 71  | ggcacgtctgattgcatgaaggttcagtgctcctagttccctccccagggaaaaac | gaca | 130 |
|       |   |     |                                                          |      |     |
| RTwt  | : | 121 | cgggagctggccaagacctctctctgggtgatgagcctaagggtgtgtgtgtagg  | gccc | 180 |
|       |   |     |                                                          |      |     |
| RTM26 | : | 131 | cgggagctggccaagacctctctctgggtgatgagcctaagggtgtgtgtgtagg  | gccc | 190 |
|       |   |     |                                                          |      |     |
| RTwt  | : | 181 | ctatgcttgcacactggggatcagacctctaccttcacccatgagg           |      | 226 |
|       |   |     |                                                          |      |     |
| RTM26 | : | 191 | ctatgcttgagggtggggatcagacctctaccttcacccatgagg            |      | 236 |

Figure 3

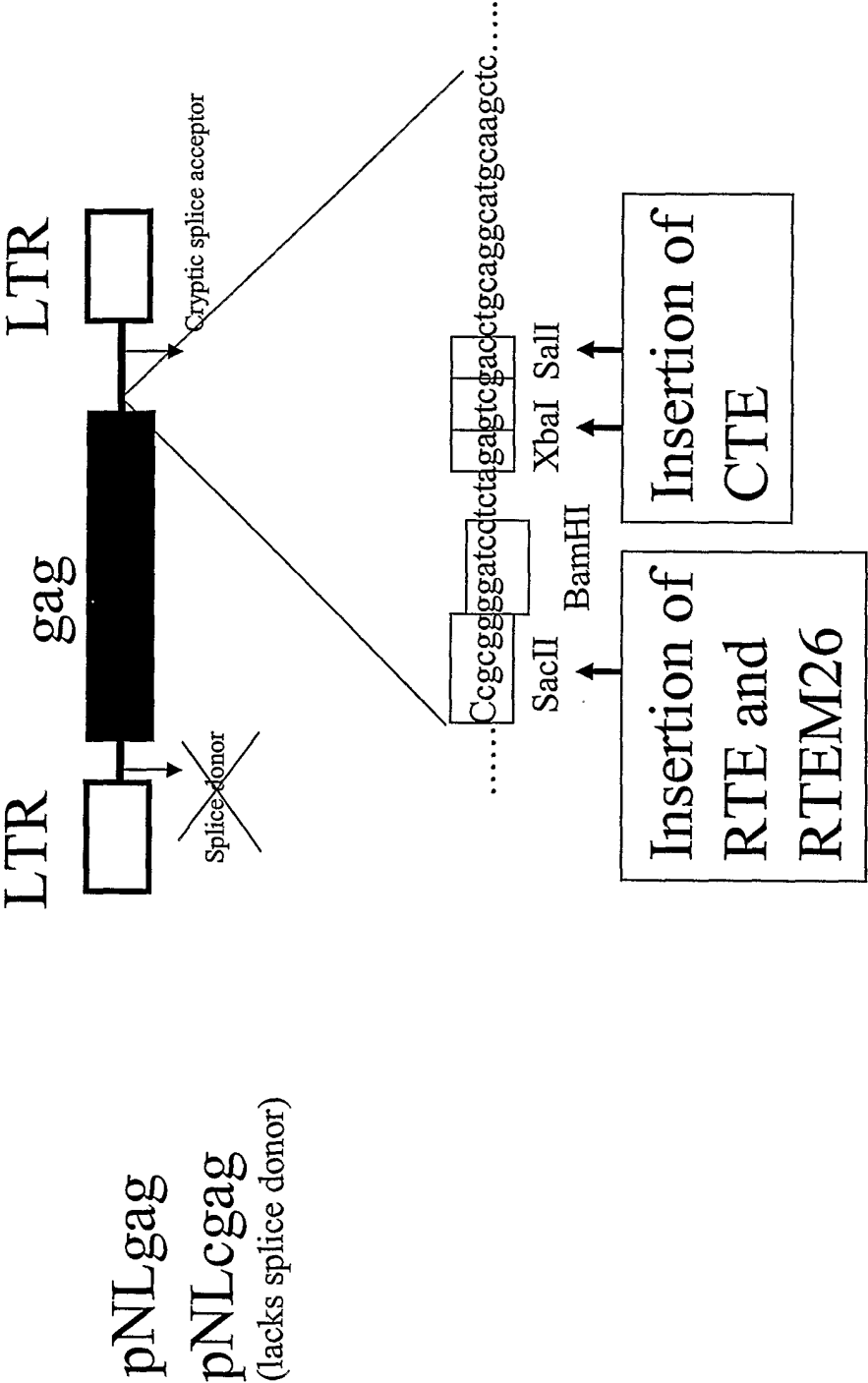


Figure 4

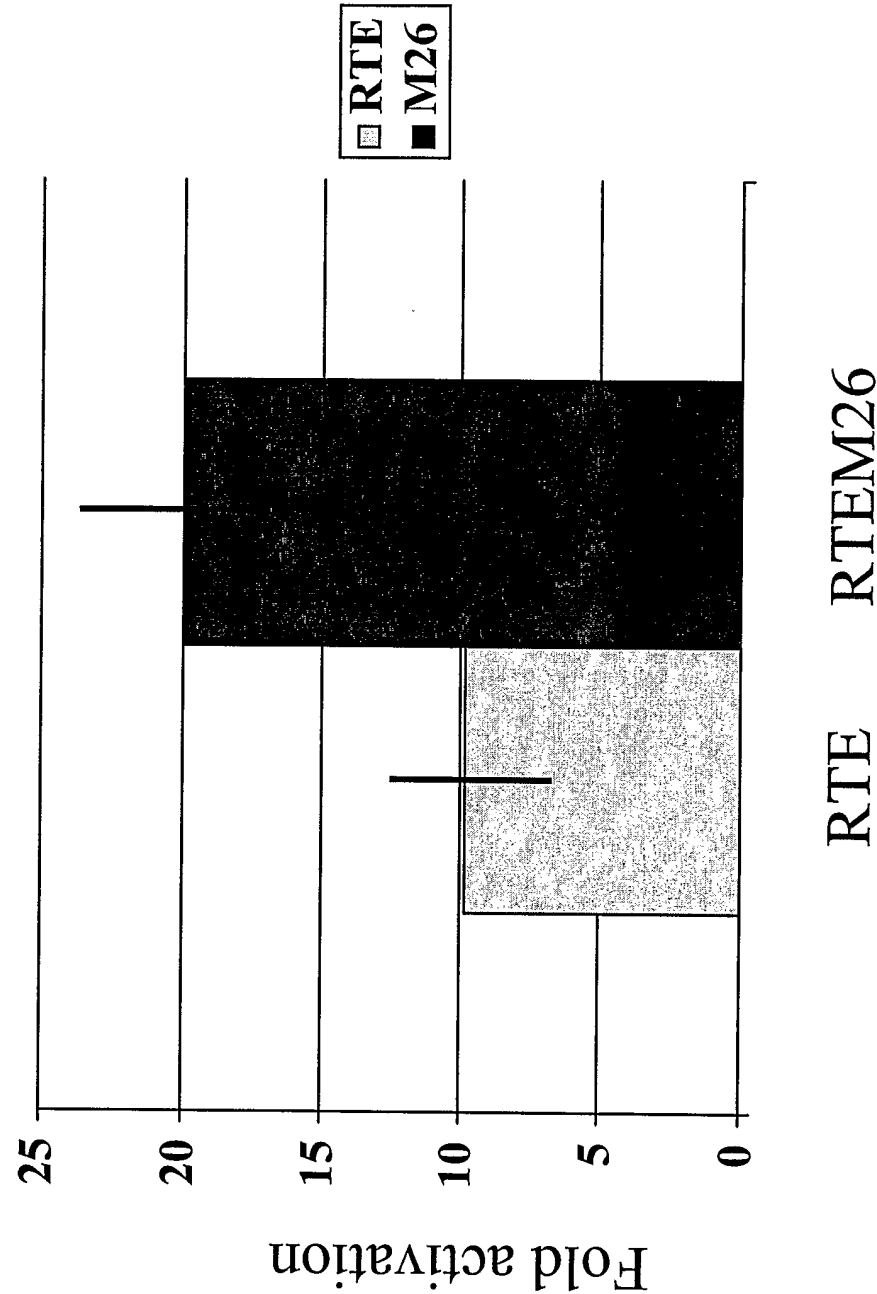


Figure 5

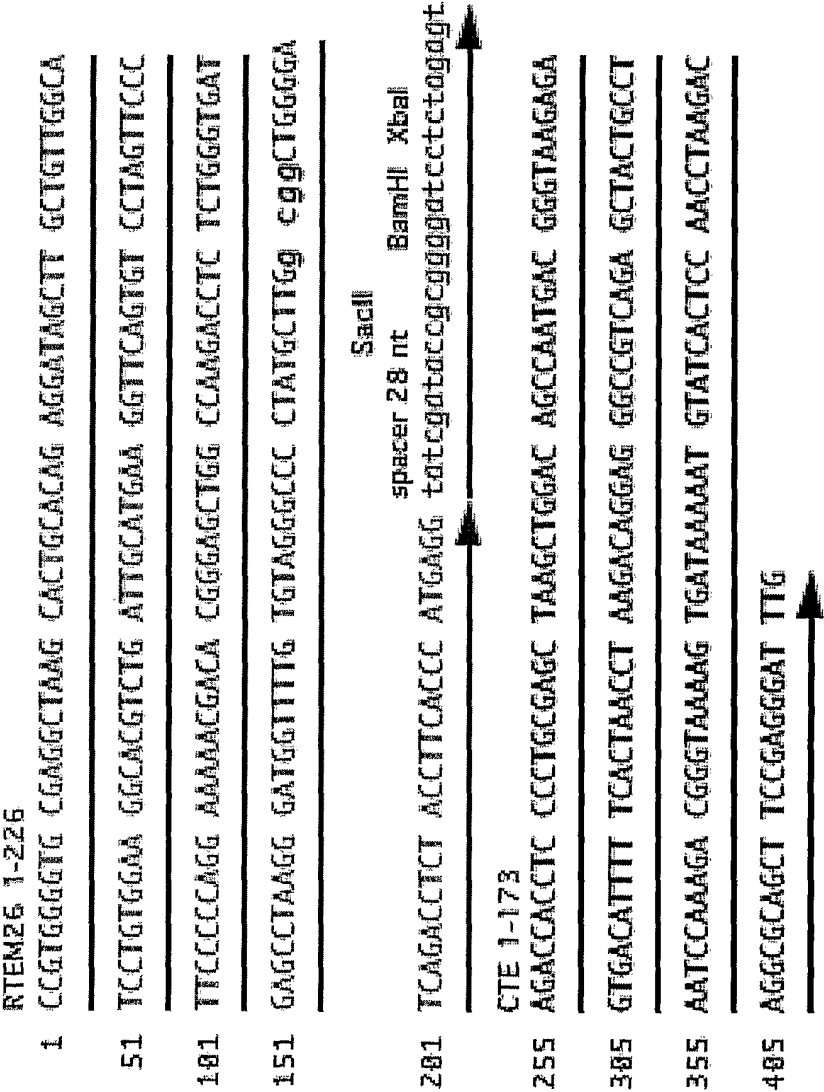


Figure 6

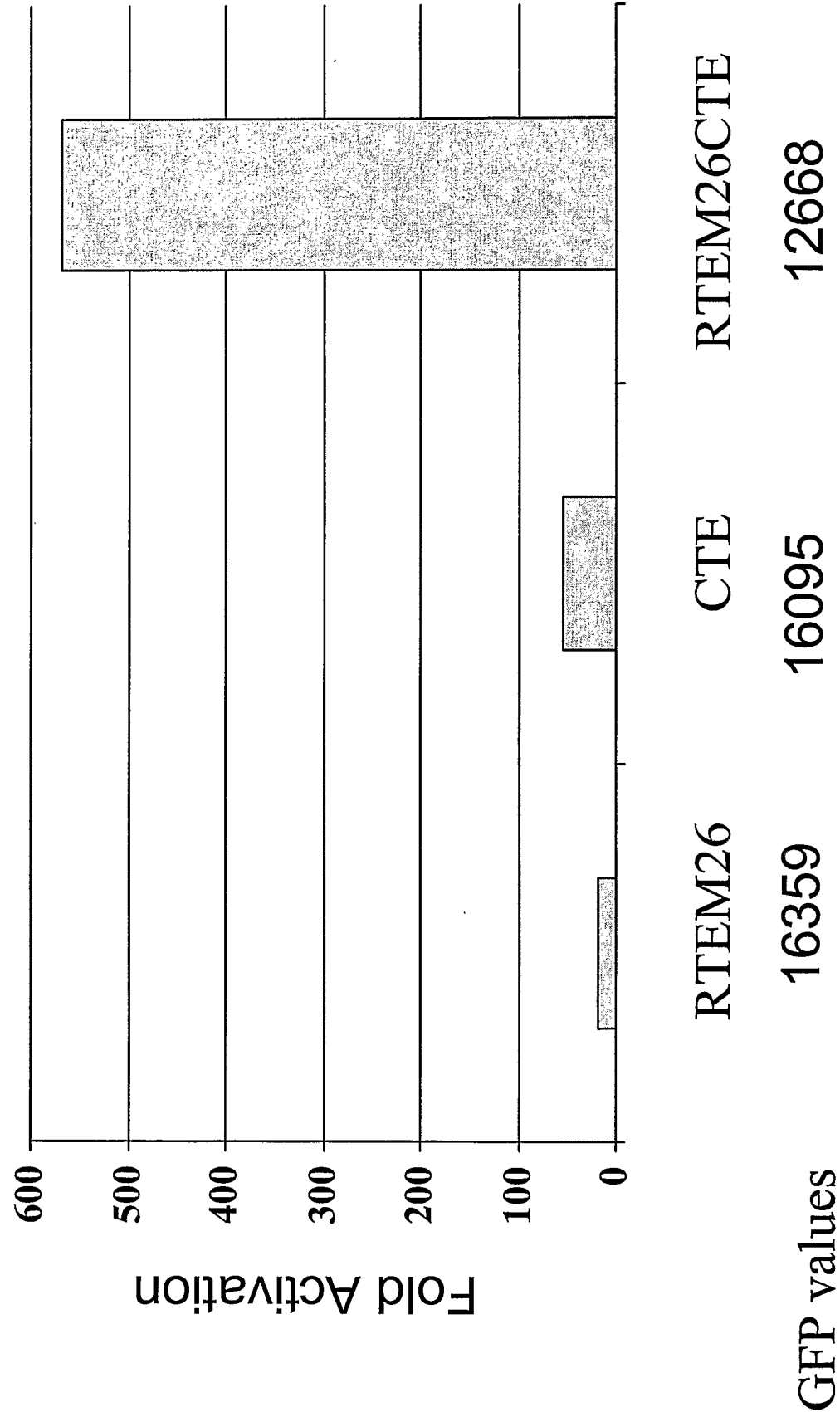


Figure 7

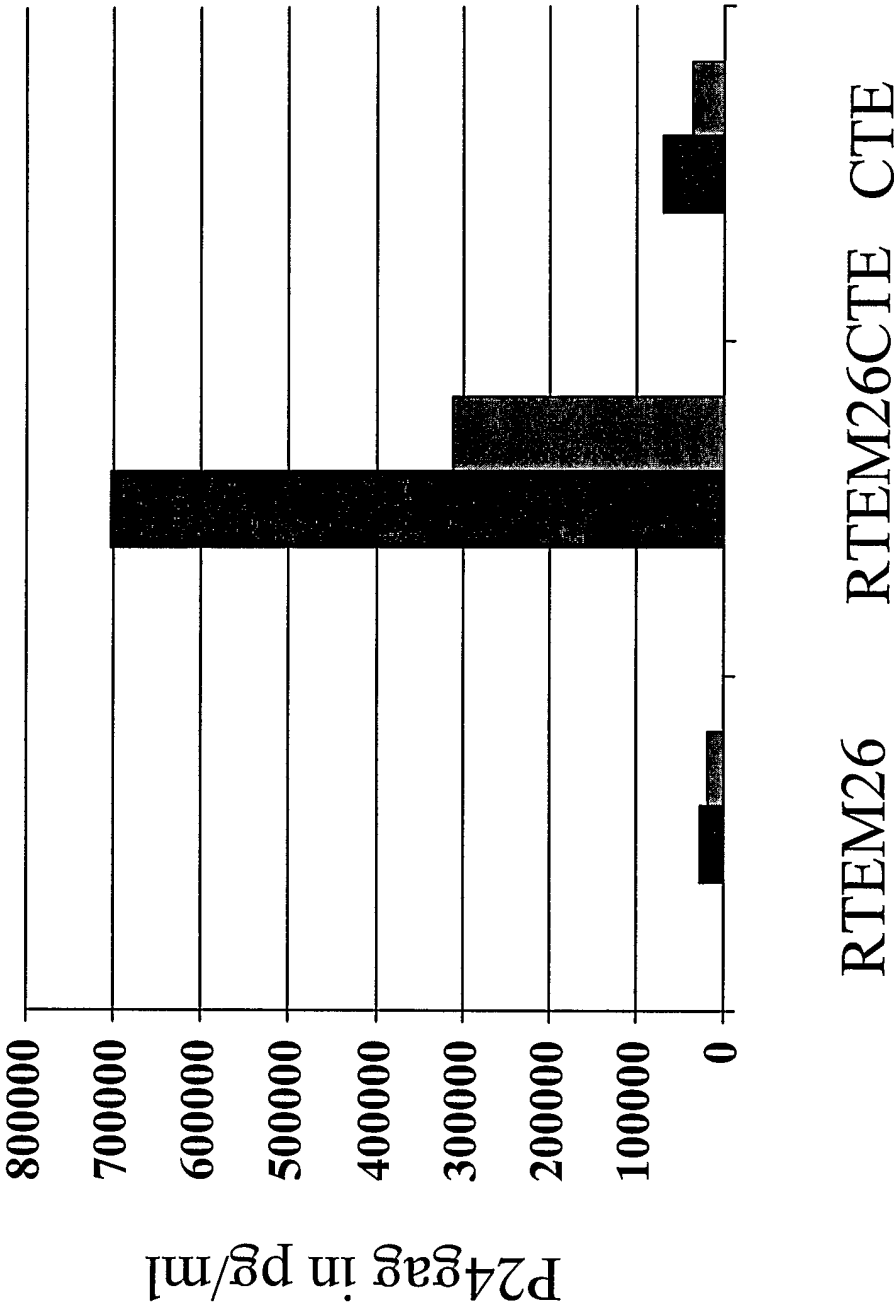




Figure 8

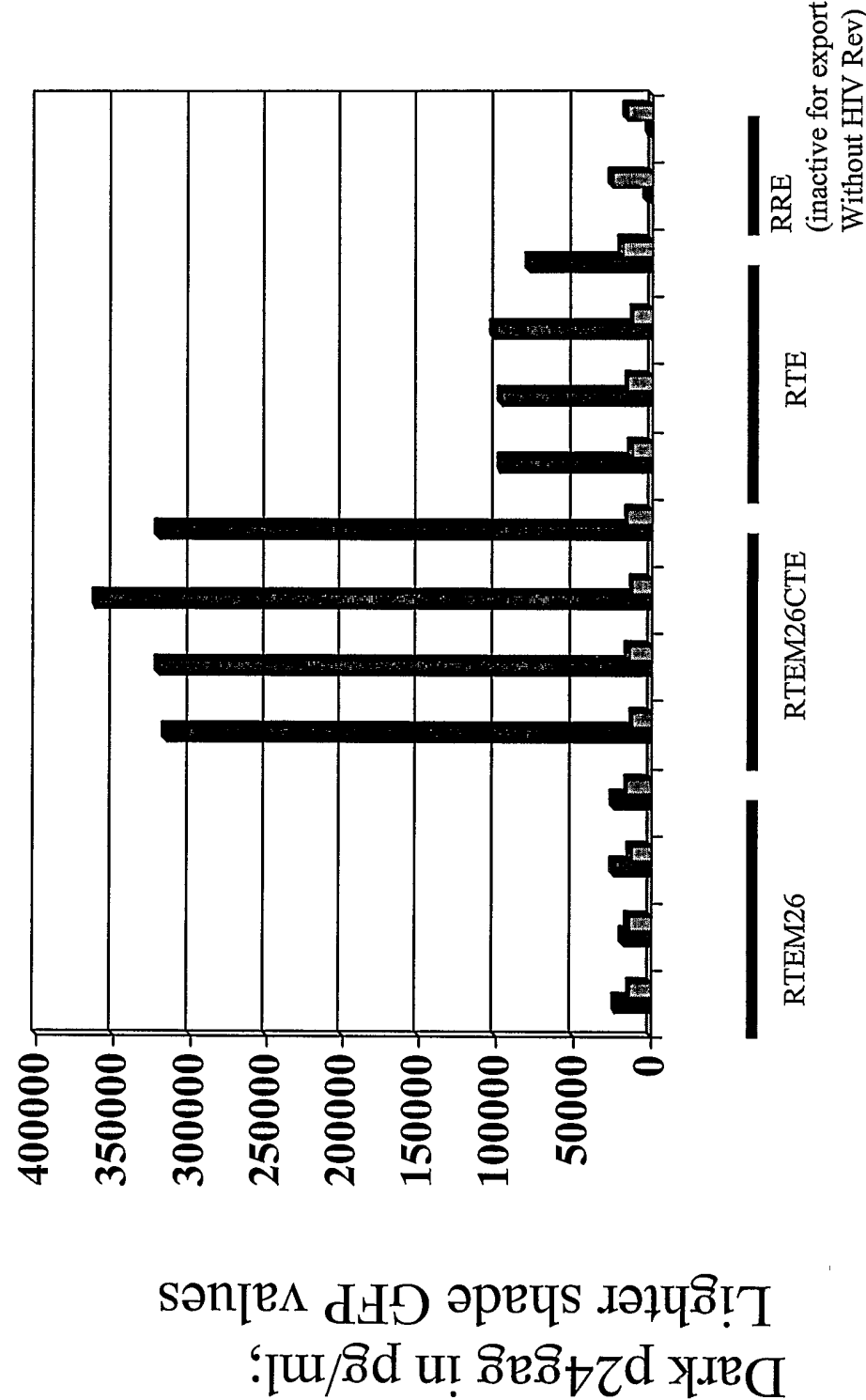


Figure 9

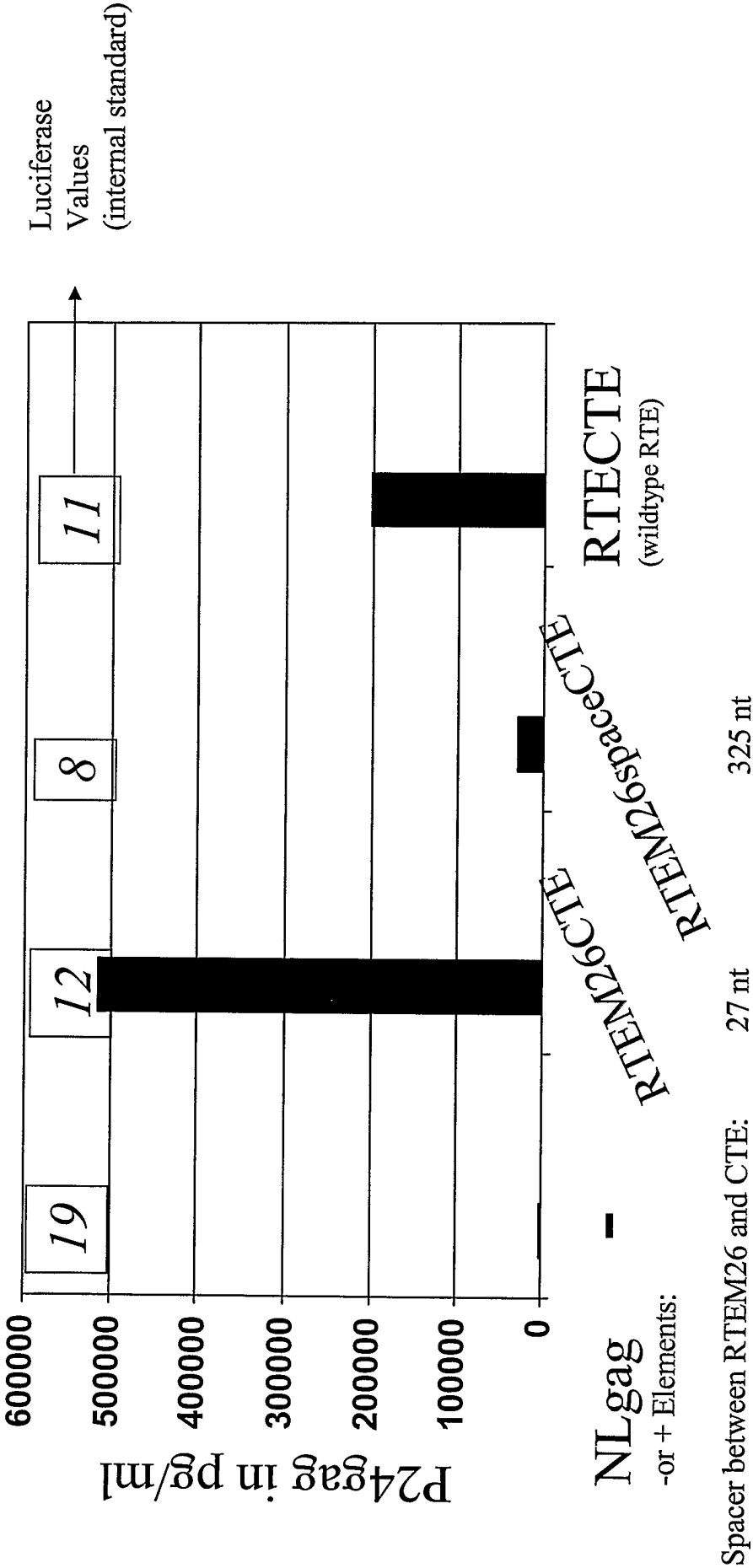
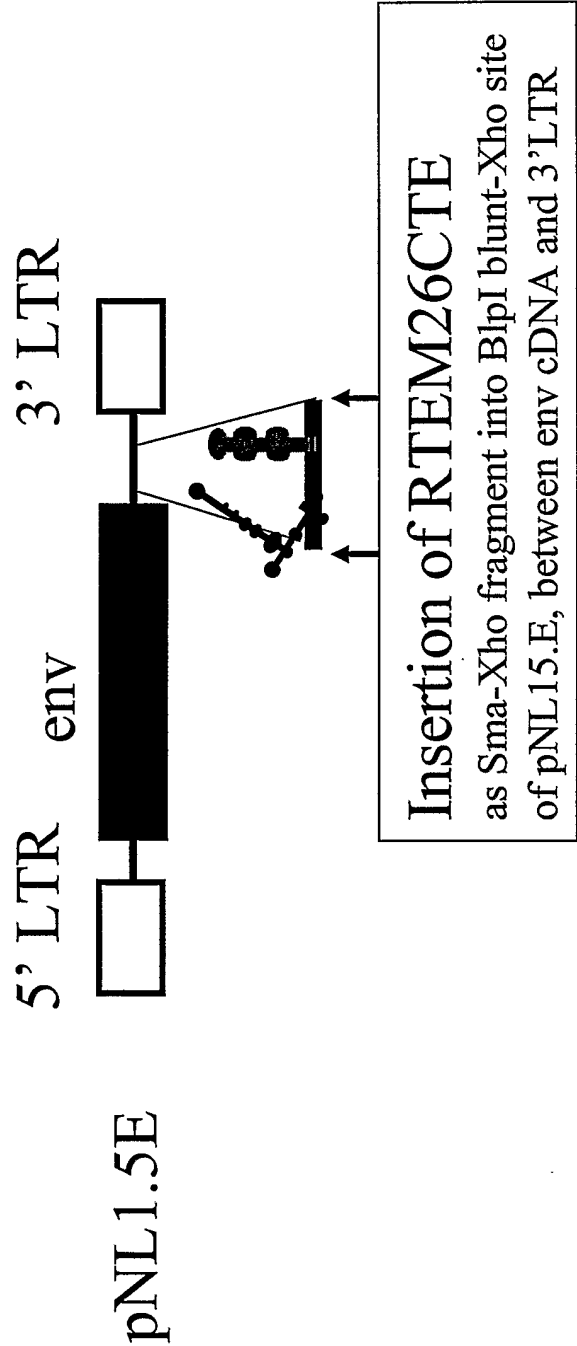


Figure 10A



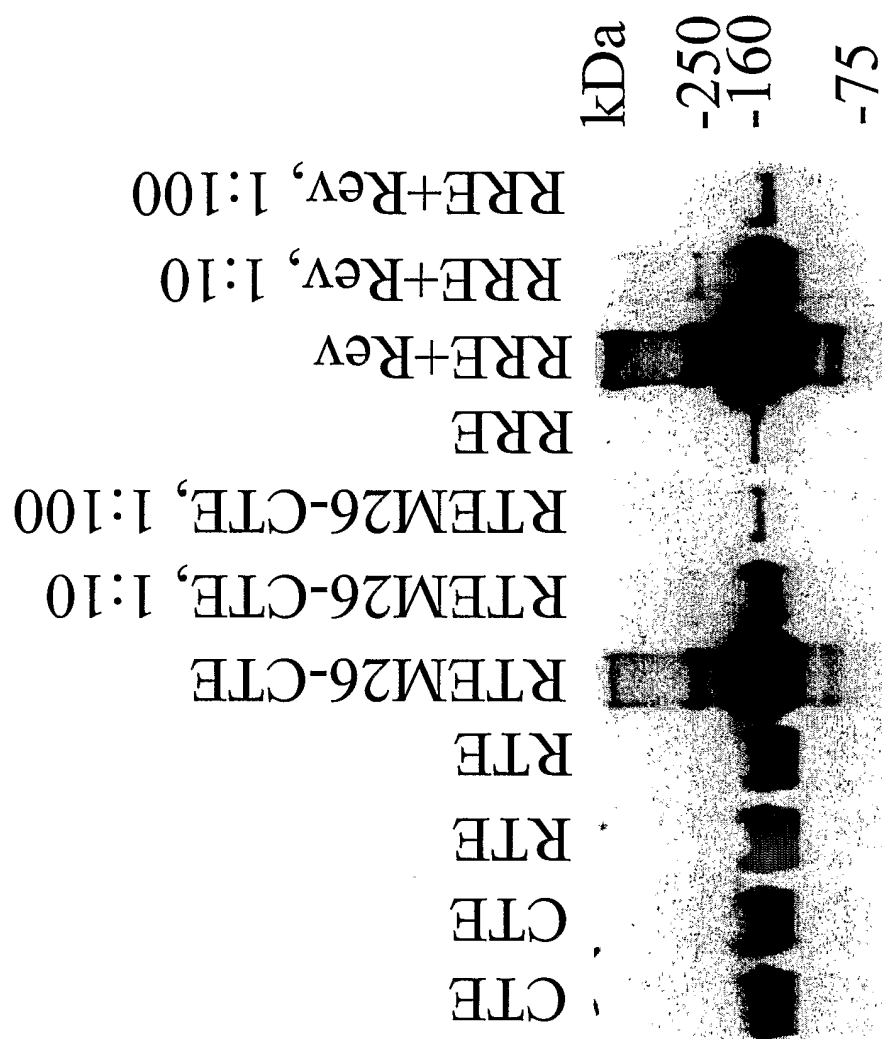


Figure 10B

4-12% PAGE, HIV-1 env antisera

Figure 11A

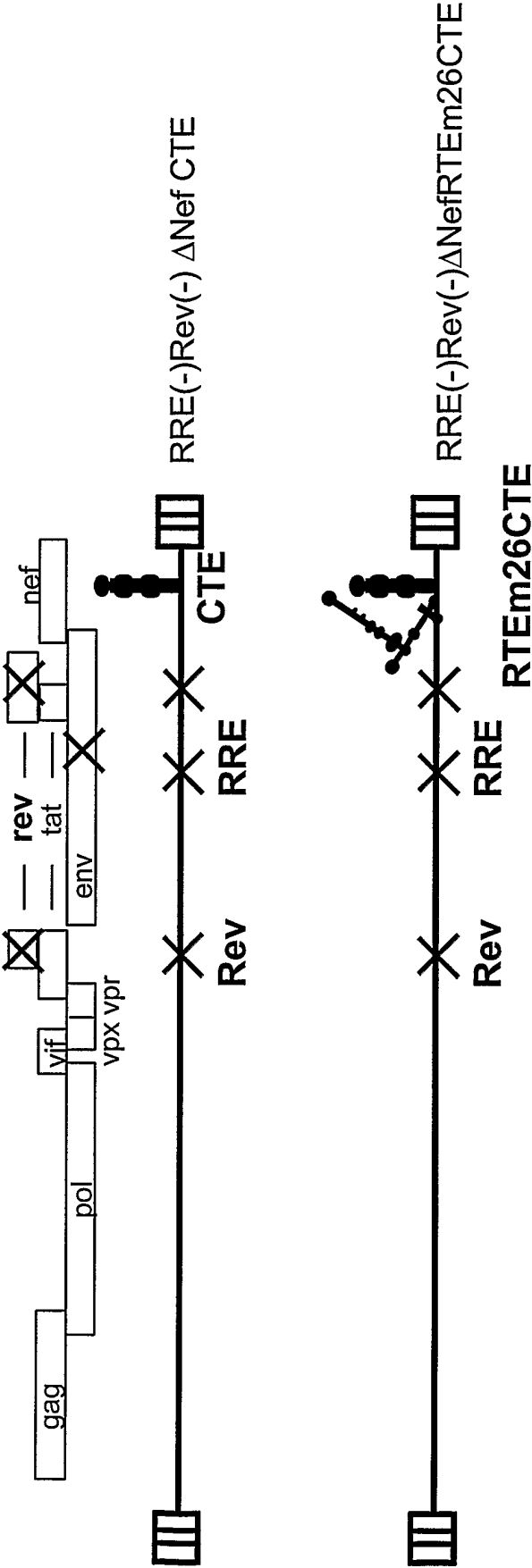


Figure 11B

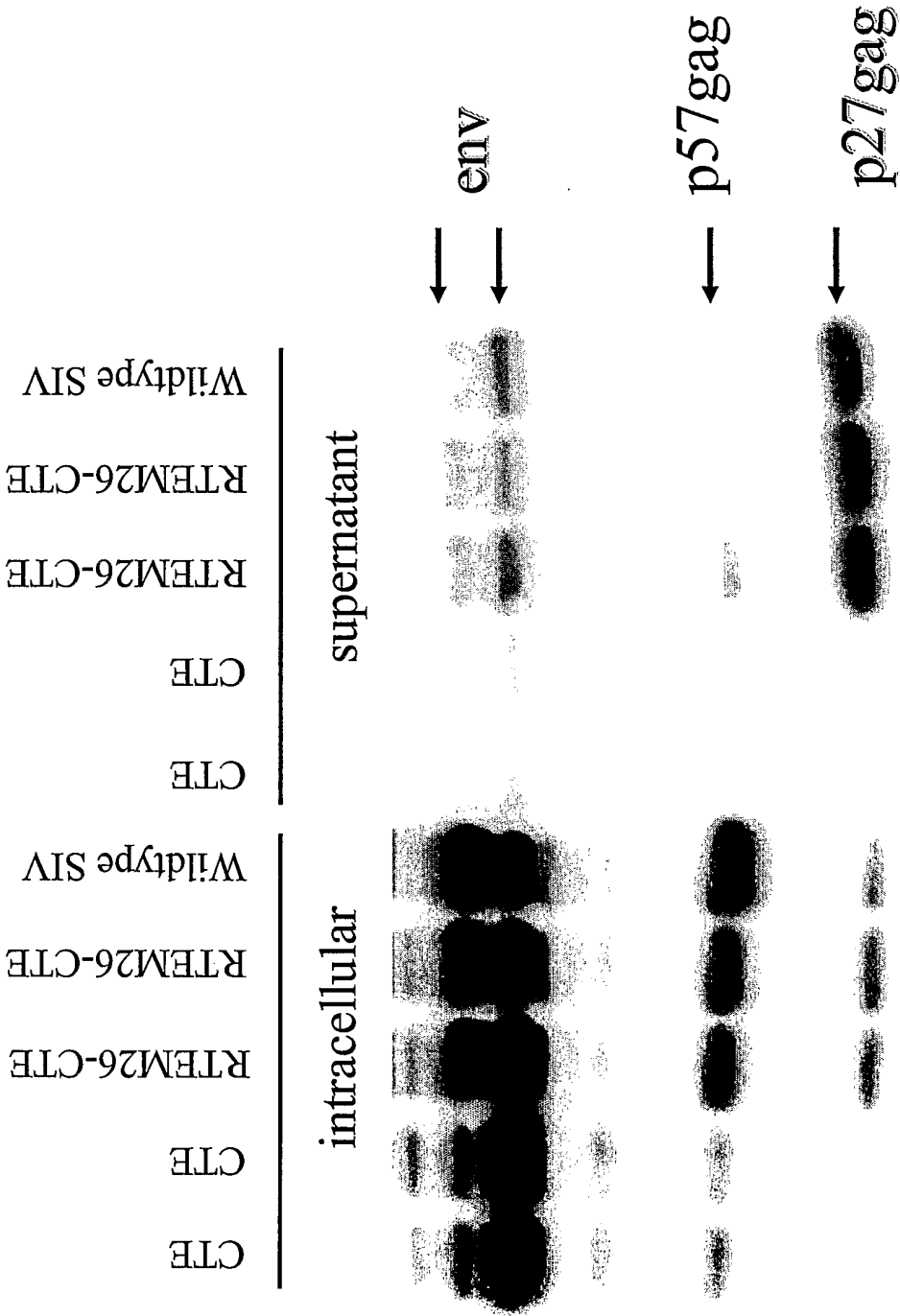
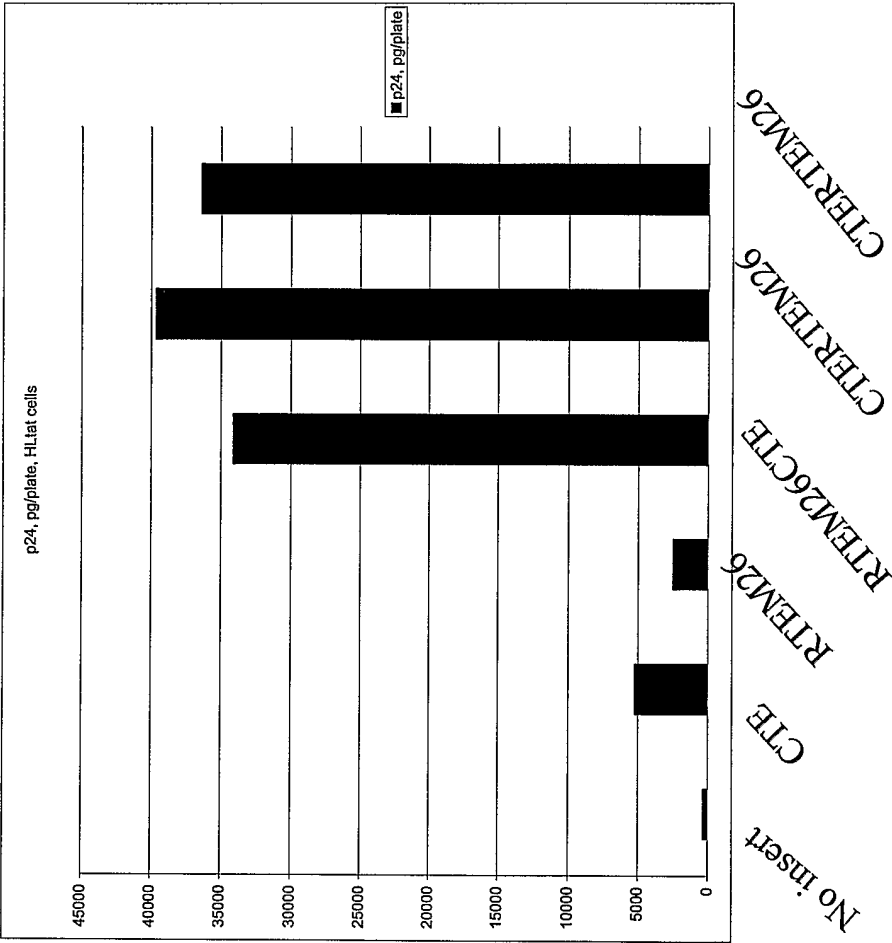
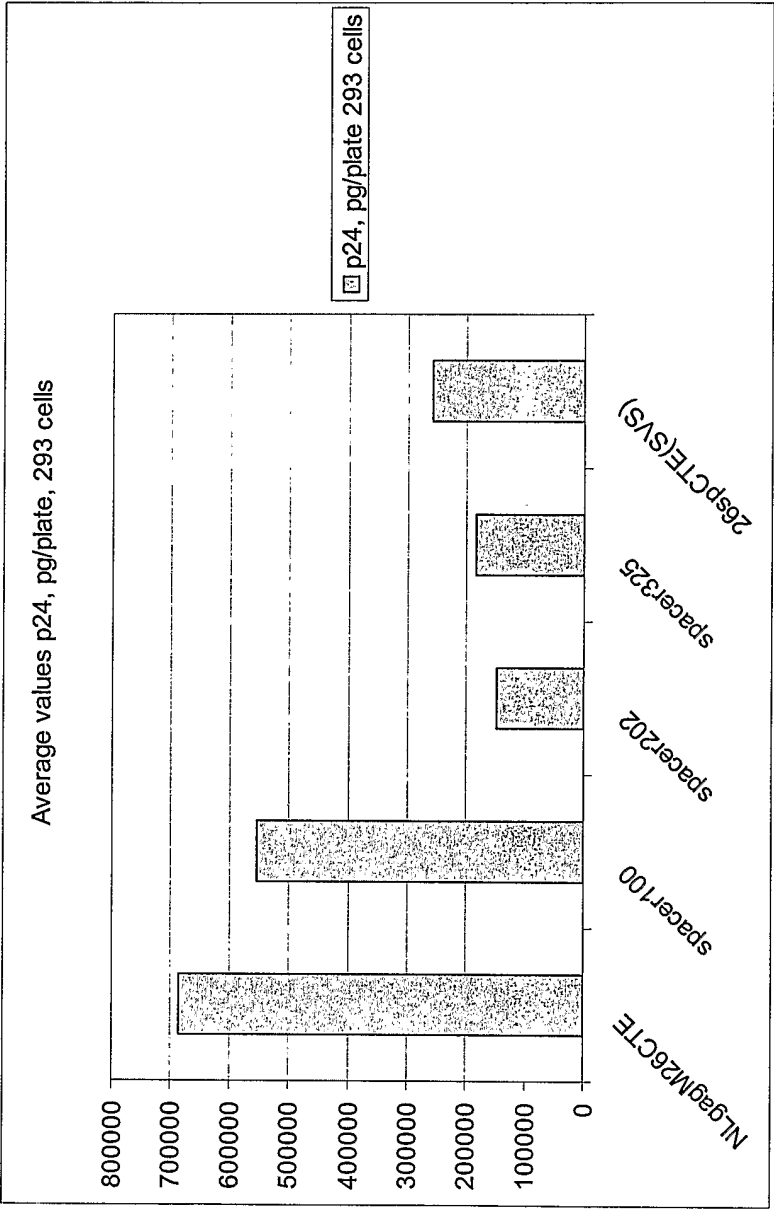


Figure 12



pNLgag  
with

Figure 13



Spacer 28 100 202 325\* 325\* nt

\* Different sequence



Figure 14

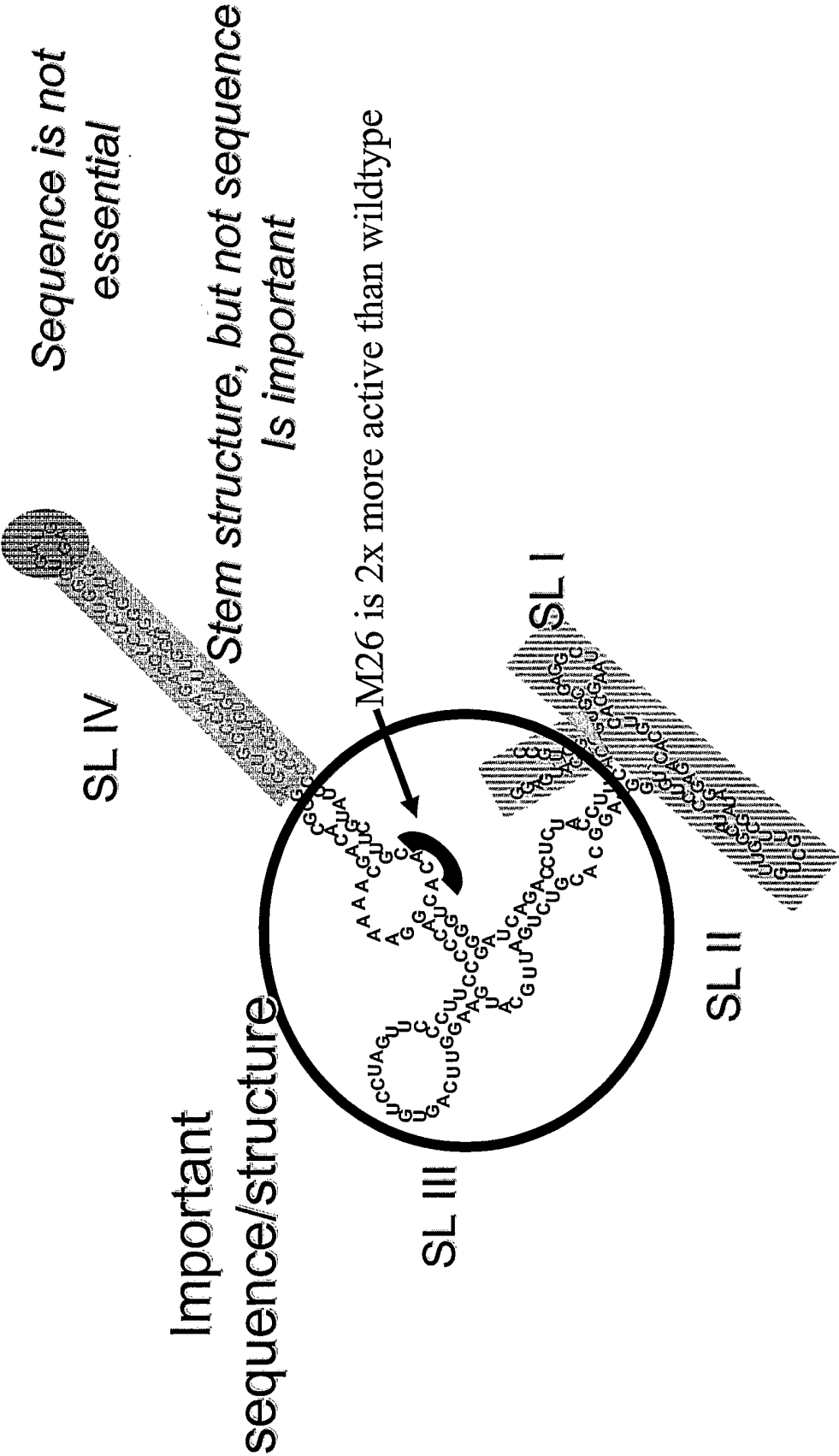
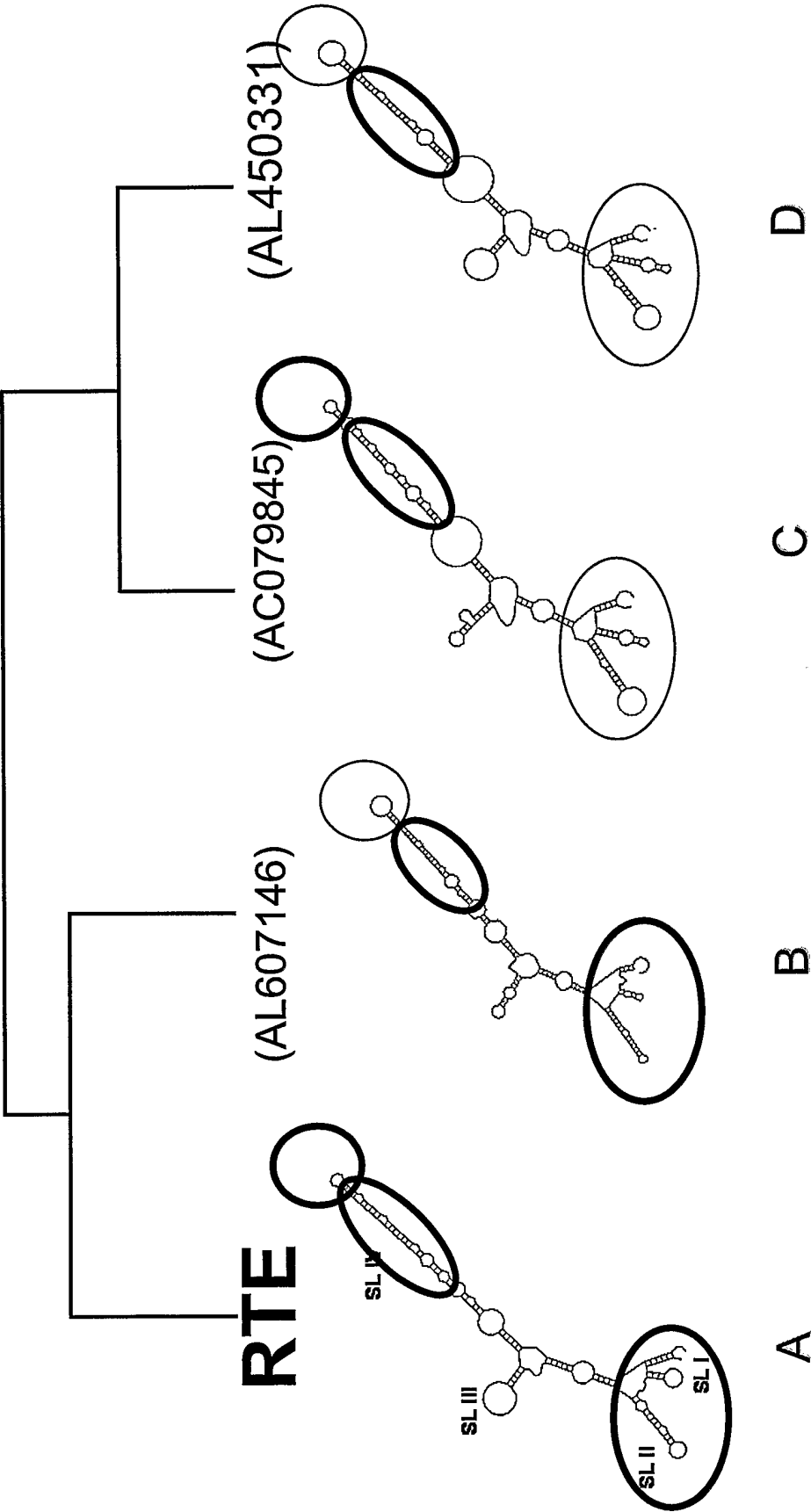


Figure 15A



|                   |   |                              |    |                                          |
|-------------------|---|------------------------------|----|------------------------------------------|
| consensus A (RTE) | 1 | CCGTGGGGTGGGAGGCTAAGCACTGCAC | 62 | AGAGGATAGCTTG A CTGTTGGCATCCTGTGGAAGG    |
| consensus B       |   | k a t ta                     |    | t tc g agctgt a rt c g                   |
| consensus C       |   | ga a a t ac                  |    | gtag ag c ctgcg c tawaatga c tattcta g a |
| consensus D       |   | ga a a t a                   |    | atgg a c ctgcg c tata tga c tattcta g a  |

SL I

|                   |    |                                                                        |     |       |
|-------------------|----|------------------------------------------------------------------------|-----|-------|
| consensus A (RTE) | 63 | CAYGTCGTGATTGCATGAAGGTTCACTGCTGCTACTTCCTTCCCCCAGGAAAAAACGACACGGGAGCTGG | 131 | g     |
| consensus B       |    |                                                                        |     | a     |
| consensus C       |    | atc t a                                                                |     | c a a |
| consensus D       |    | atc t                                                                  |     | a     |

SL III

|                   |     |                     |     |                                             |
|-------------------|-----|---------------------|-----|---------------------------------------------|
| consensus A (RTE) | 132 | CCAAGACCTCTCTGGGTGA | 194 | TGAGCCCTAAGGGATGGTTTGTGTAGGGCCCTATGCT TGCAC |
| consensus B       |     | t g gtr             |     | a tcaa cc ac t yw                           |
| consensus C       |     | g c                 |     | gg a ga a c t tacc                          |
| consensus D       |     | t g gttg            |     | a c aa cc ac t t tacc a                     |

SL IV

|                   |     |                                     |     |              |
|-------------------|-----|-------------------------------------|-----|--------------|
| consensus A (RTE) | 195 | ACTGGGGATCAGACCTCTACCTTCACCCCATGAGG | 226 | ga           |
| consensus B       |     |                                     |     | tt           |
| consensus C       |     | tt                                  |     | t c t tcatta |
| consensus D       |     | tt                                  |     | t c t tcatta |

Figure 15B

|          |   |             |    |     |   |      |    |                   |   |
|----------|---|-------------|----|-----|---|------|----|-------------------|---|
| RTE      | 1 | CCGTGGGGTGC | at | tig | c | SL I | 62 | TGGCATCCTGTGGAAGG | a |
| AL607146 |   | ga a a t    | ac |     |   |      |    | gca tcc a         | a |
| AC079845 |   | ga a a t    | ac |     |   |      |    | c tattcta         | a |
| AL450331 |   |             |    |     |   |      |    | c tattcta         | a |

SL II

|          |    |                                       |   |     |    |   |        |     |                       |   |   |   |   |   |
|----------|----|---------------------------------------|---|-----|----|---|--------|-----|-----------------------|---|---|---|---|---|
| RTE      | 63 | CACGTCGTGATTGCATGAAGGTTGAGTGTGCTAGTTC | g | a   | gt | c | SL III | 131 | AAAAACGACACGGGAGCTGGC | t | a | g | a | t |
| AL607146 |    | a gat                                 | g | a   | gt | c |        |     |                       |   |   |   |   |   |
| AC079845 |    | t atc                                 | a | t a | gt |   |        |     |                       |   | a | c | a |   |
| AL450331 |    | t atc                                 | t |     |    |   |        |     |                       | c |   | a | t |   |

|          |     |                    |                                                |                          |
|----------|-----|--------------------|------------------------------------------------|--------------------------|
| RTE      | 132 | CAAGACCTCTCTGGGTGA | TGAGCCTAAGGGATGGTTTTGTGTAGGGCCCCCTATGCTTGCACAC | 194                      |
| AL607146 |     | g gttg             | a aagcctg                                      | a c aa cc ac t ttc       |
| AC079845 |     | g c                |                                                | gg a ga a c t t a c a    |
| AL450331 |     | g gttg             | a aagcctg                                      | a c aa cc ac t t t a c a |

SL IV

|          |     |                                  |              |
|----------|-----|----------------------------------|--------------|
| RTE      | 195 | TGGGGATCAGACCTCTACCTTCACCCATGAGG | 226          |
| AL607146 |     | a tc                             | t t aa       |
| AC079845 |     | tt                               | t c t tcatta |
| AL450331 |     | tt                               | t c t tcatta |

Figure 15C