(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2012 (01.11.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/149566 A2

(51) International Patent Classification:
A61K 48/00 (2006.01) *A61P 31/18* (2006.01)
A61K 31/7105 (2006.01) *A61P 31/12* (2006.01)
A61K 31/7088 (2006.01)

(21) International Application Number:
PCT/US2012/035878

(22) International Filing Date:
30 April 2012 (30.04.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13/096,626 28 April 2011 (28.04.2011) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

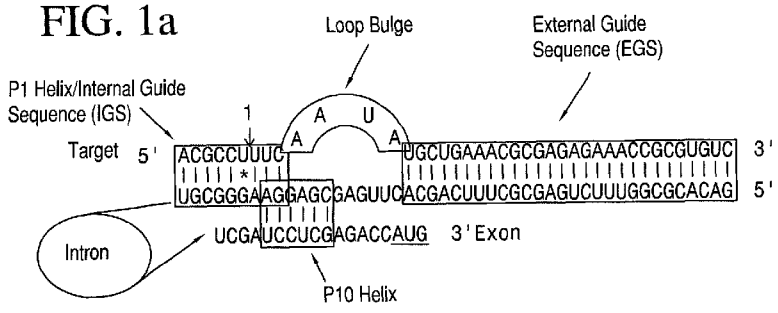
Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2012/149566 A2

(54) **Title**: ANTI-HIV GROUP I INTRONS AND A USES THEREOF IN TREATING HIV INFECTIONS

FIG. 1a



(57) **Abstract**: Described is a unique class of antiviral molecule that can be applied to control and eliminate HIV infection in patients using myeloablation therapies and replenishment with transformed bone marrow stem cells programmed to express the antiviral molecule. These anti-viral molecules target the HIV genome in a highly conserved domain, and when expressed in cells prior to infection will cause the cell to die upon infection with HIV. Cell death insures no proliferation of new virus. Reconstituting the immune system with cells expressing these antivirals prevents re-establishment of HIV infection from reservoirs in the re-established lymphocyte and macrophage populations. Over time, reservoirs will be depleted entirely, effectively eliminating the virus. In effect, this new type of antiviral can be used to cure HIV infections.

ANTI-HIV GROUP I INTRONS AND A USES THEREOF IN TREATING HIV INFECTIONS

Statement of Government Support

[0001] The United States federal government owns rights in the present technology as research was supported by funds from National Institutes of Heath Grant # 777.

Field of the Invention

[0002] The present invention relates to the filed of pharmaceutical preparations and methods for treating and/or inhibiting human immunodeficiency virus (HIV) infection.

Background of the Invention

[0003] At present there is no generally applicable cure for HIV infections of humans. While some development of anti-viral drugs and therapies exists, these have not yet proven capable of eliminating the virus from the infected patient. This is in large part due to the establishment of infected tissue reservoirs for the virus in which the virus may remain relatively quiescent for long periods of time, continuously shedding small amounts of virus into the patient to maintain or reestablish infection. A need continues to exist in the Art to cure a patient of the HIV infection.

[0004] While highly active anti-retroviral therapy (HAART) provides some suppression of HIV infection and allows restoration of immune function, effectively circumventing the decline into AIDS, HAART treatment alone cannot provide a cure for the infection. In addition, there remain several problems with this approach, including the expense of long term treatments, the continuing contagiousness of the patient, development of escape mutations, and toxicity associated with long term drug treatments. Discontinuation of HAART allows rapid rebound of the infection. Similarly, rebound of infection may occur through evolution and selection of quasi and mutated species resistant to HAART. Both of these viral rebounds result from an inability to completely clear the virus from infected patients due to the presence of persistently infected, long-lived lymphocyte or macrophage cell populations that serve as reservoirs for the virus (see Blankson et al., 2002).

[0005] Alternative approaches utilizing transplantation of *ex vivo* genetically transformed lymphocyte and macrophage lineage cells or progenitor stem cell populations are being explored as a means of suppressing HIV infection in susceptible cell populations (See Rossi et al., 2007). One major drawback to many of these approaches is their focus on suppressing the production of new virus in re-implanted cells while still allowing these cells to be re-infected. This approach allows the re-establishment and perpetuation of the very reservoirs that permit the evolution of quasi species of the virus, and allows escape mutants with these mutations to expand.

[0006] The first confirmed catalytic RNA, the cis-splicing intron of *T. thermophila* pre-rRNA, termed 'ribozyme' to describe the RIBO nucleic acid-based enzyme, was reported by Kruger et al. (1982). This intron excised itself from the highly purified mature rRNA in a solution of magnesium and guanosine in a cell-free system. Later, this intron was configured to splice together RNA on two separate molecules by two successive trans-esterification reactions (Sullenger & Chec, 1994).

[0007] The trans-splicing Group I intron reaction targets an RNA molecule through the use of antisense guide sequences that hybridize with the target RNA and permit cleavage at a specific uracil, releasing the downstream sequence (see Figure 1). This step frees a 3' OH downstream of the uracil cleavage point to act as a nucleophile which carried out the covalent joining of the upstream target RNA fragment to an intron-associated 3' exon, resulting in a new, continuous RNA molecule. This reaction can be designed to generate a new contiguous open reading frame, leading to a transcript molecule that encodes a protein product that is present if and only if splicing has occurred.

[0008] In a trans-splicing reaction, two separate segments of the intron are utilized to specify the RNA sequence the ribozyme will target. The internal guide sequence and external guide sequence (IGS and EGS, respectively) are each complementary to a segment of the target. The IGS is limited in size to roughly 9 base pairs near the target uracil, and forms what is termed the P1 helix with the target, where the reaction will eventually occur. The EGS can be of nearly any length and forms a transient helix downstream of the target

uracil. A longer EGS will increase the specificity and affinity of the intron towards its target RNA (Kohler et al., 1999).

[0009] The trans-splicing reaction is catalyzed by the P10 helix that is formed by the 3' end of the intron in the vicinity of the splicing reaction to guide the 3' exon to the proper ligation point. This step is vital to the second step of the reaction as it enables the free 3' OH of the cleavable uracil to attack the phosphate backbone upstream of the 3' exon, allowing covalent joining of the 3' exon to the upstream cleavage product to create a new, seamless mRNA suitable for translation.

[0010] Group I intron trans-splicing has been used in a number of applications including repair of mutant B-globin mRNA (Byun et al., 2003), restoration of wild-type p53 activity in three cancerous cell lines (Lander et al., 2001), re-establishment of the function of the canine skeletal muscle chloride channel (Waterson et al., 2002), and induction of p16 activity in a pancreatic cell line (Kastanos et al., 2004). The trans-splicing group I intron has proven to be an effective anti-cancer therapy in model systems. Researchers were able to cause the cell-specific death of human colon cancer cells by targeting an mRNA coding for the carcinoembryonic antigen utilizing HSV-tk as a 3' exon followed by ganciclovir treatment (Jung & Lee, 2006). This same group, using similar methods, achieved group I intron catalyzed trans-splicing of the liver-cancer upregulated α-fetoprotein (AFP) in human liver cancer cells (Won & Lee, 2007) and the mouse homologue of the cancer associated cytoskeleton-associated protein 2 in mammalian cells (Kim et al., 2007). Also reported is the cell-specific cytotoxicity induced via generation of diphtheria toxin A (DTA), or ganciclovir/herpes simplex V thymidine kinase (HSV-tk)-induced apoptosis in cells expressing the tumo. These Group 1 trans-splicing introns r associated hTERT subunit of telomerase (Jung et al., 2005; Kwon et al., 2005), and trans-splicing of the hepatitis C virus internal ribosome entry site (HCV-IRES) (Ryu et al., 2003).

[0011] Despite these and other reports, a need continues to exist in the medical arts for more effective and long-lasting treatments for human immunodeficiency virus (HIV) infection, and in halting the progression of the infection to AIDS.

Summary of the Invention

[0012] The present invention provides for methods and preparations that may be used in the treatment and control of human immunodeficiency virus infection, as well as the disease known as AIDS.

[0013] In one aspect, the invention provides for the use of specifically designed Group I trans-splicing introns that may be used as transgenic anti-viral (anti-HIV) agents. These agents effectively protect an animal against viral infection by human immunodeficiency virus using a "death-upon-infection" (DUI) approach. The effectiveness of this anti-viral strategy is demonstrated by the presently disclosed design of introns that attack conserved human immunodeficiency virus viral genomic RNA sequences. In particular, the present invention demonstrates the utility of these introns for the human pathogen, human immunodeficiency virus (HIV).

[0014] A unique class of anti-viral molecules is provided that may be used in clinical treatment methods to control and eliminate HIV infection in patients. In some embodiments, myeloblation therapies and replenishment with transformed stem cells programmed to express the antiviral (anti-human immunodeficiency virus) molecule is employed as part of the therapeutic method. By way of example, the transformed stem cells are transformed bone marrow cells. The antiviral molecules target the HIV genome in a highly conserved domain, and when expressed in cells prior to infection, will cause the cell to die upon infection with HIV. The death of HIV infected cells insures no proliferation of new virus will occur. As part of the treatment method, the patient will then be subjected to a treatment that provides for the reconstitution of the patient's immune system with cells expressing the antiviral molecules. The now reconstituted immune system of the patient thus prevents the re-establishment of HIV infection, such as re- infection that might otherwise occur from virus infected cells within reservoirs in the treated animal because of the re-established lymphocyte and macrophage populations. It is expected that over time, any reservoirs of infected cells will be depleted entirely, effectively eliminating the virus, thus providing a cure and/or method for inhibiting HIV infections.

[0015] A trans-splicing Group I intron approach is provided for suppression of HIV-infections in both humans and animals (e.g., mice, etc.), and presents an effective means of suppressing HIV infection.

[0016] The present invention is another aspect provides an *ex-vivo* transgenic procedure for treating infection by establishing modified (transformed) lymphocyte and macrophage stem cell populations that are incapable of supporting HIV infection *in vivo*, and providing the modified lymphocyte and/or macrophage stem cell populations to an infected individual/animal. In some embodiments, the lymphocyte and/or macrophage stem cell population is prepared from a population of cells obtained from the patient to be treated, and then transformed according to the present procedures prior to administration to the patient being treated.

[0017] In another aspect, the invention provides modified (transformed) lymphocyte and macrophage stem cell populations that are incapable of supporting HIV infection in vivo.

[0018] In yet another aspect, the invention provides Group I introns that attack HIV genomic or mRNAs, and that splice to these target RNA molecules a 3' exon encoding an apoptosis-inducing gene. HIV susceptible cells expressing this anti-HIV group I (αHIV-Grpl) intron will be effectively primed to undergo apoptotic cell death in response to infection by the HIV virus, rather than surviving and producing new progeny virus. This strategy will significantly reduce and/or eliminate, the possibility of generating escaping mutations. The effectiveness of this anti-HIV strategy may be verified in transformed cell cultures using splicing assays to demonstrate the activity of the αHIV-Grpl intron, and virus titration assays to assess the productivity of the transformed cell cultures. Apoptosis assays may be used to verify the activity of the trans-spliced apoptosis-inducing product.

[0019] An in vivo animal model (mouse) will be used to demonstrate the utility of the present invention for providing a method and composition useful in the inhibition and/or treatment of an active HIV infection *in vivo*. This model permits examination of the parameters for application of this DUI strategy *in vivo* to eliminate HIV infection of a host, and ultimately cure the disease.

[0020] The present invention provides a means of suppressing HIV infections that will lead to an effective cure for this disease. By engineering target cell populations to undergo apoptotic cell death upon infection with HIV, instead of permitting the establishment of an infectious cell condition, the virus is divested of a means of re-amplifying in genetically modified, implant-derived tissues. Using procedures standard to the clinical field, patient-derived (autologous) bone marrow stem cells will be transformed *ex vivo* to express the presently disclosed anti-HIV effector gene. These transformed stem cells will then be re-implanted into the patient. The transformed stem cells will then expand *in vivo* with HAART suppression of the HIV infection. After sufficient expansion, removal of HAART and re-infection of these re-implanted cells would not result in re-establishment of the infection, but would result in a continual reduction of virus load through apoptotic death of these re-infected cells. Continued expansion of the remaining uninfected re-implanted cells eventually results in the replacement of susceptible cells and even reservoirs with virus free cells, thus curing the disease.

[0021] The utility of the present methods for providing a treatment for inhibiting and/or curing human immunodeficiency virus is supported in part by a study of a CCR5Δ32/Δ32 hematopoietic stem cell transplantation (SCT) to treat Acute Myeloid Leukemia in an HIV-1 infected patient (Allers et al., 2010). Following a protocol that involved depletion of the patient's own hematopoietic cells, the SCT resulted in replacement of patient hematopoietic cells with donor-derived lymphocyte and macrophage lineage cells, all CCR5Δ32/Δ32, and led to long term eradication of the HIV infection. However, this procedure does not provide for use of autologous cells, and requires finding a compatible donor that is CCR5 deficient. These drawbacks are eliminated with the present methods, which in certain embodiments provides for the use of autologous cells, and is not limited to the use of donor cells that are CCR5 deficient.

[0022] Using the Group I intron approach, the present invention provides a transgenic "death upon infection" strategy with a patient's own stem cells that will effectively accomplish long term suppression of HIV, and has the advantage of eliminating the necessity for finding a tissue compatible donor. Moreover, the Group I intron effector

transgene can be coupled as a di-cistronic RNA molecule with an IRES dependent selectable gene that would permit selective expansion and maintenance of transformed SCT-derived cells, allowing expansion of transformed hematopoietic stem cells in vivo in the absence of immune ablation therapies.

[0023] Developing a cure for HIV infections is provided in one aspect of the invention. The successful demonstration of the effectiveness of this approach for suppressing chronic virus infections revolutionizes the treatment of several chronic infectious diseases. Analogous diseases that could be approached using this strategy are the chronic viral hepatitis diseases, HCV and HBV. In this sense the development of this transgenic anti-viral Group I intron approach provides a unique platform technology that provides for a new paradigm in the use of the presently described trans-splicing ribozymes in disease treatment.

[0024] The specific design of the present introns will include a conserved nucleotide sequence within the HIV genome, such as the sequence of the upstream $tRNA^{lys3}$ primer binding site (PBS) (Amarasinghe et al. (2000). The accessibility of conserved sequences to Group 1 intron attack within a specific 540 nucleotide long target sequence encompassing the 5' terminus of the HIV genome and including the tRNA PBS and Ψ-packaging signal was examined. The Ψ-packaging signal sequence was not useful as a targeted sequence for the Group 1 intron because the targeted sequence for cleavage occurred in a complex of the stem loop structure that was not accessible to intron under normal physiological conditions.

[0025] According to one aspect, an αHIV/Grp1 intron strategy is provided that targets absolutely or at least highly conserved sequences of the HIV genome that are present in the full length genomic RNA and in each mRNA molecule produced following proviral integration and expression. In this respect, another key innovation that is designed to insure success of the approach is the focus on the tRNA primer binding sequence as a relatively immutable and abundant target, thus insuring an extremely low probability, possibly none, of evolving escape mutants.

[0026] According to another aspect, an overall strategy of cell death upon infection as a means for reducing and eliminating virus from the infected individual is provided. The body does support and even encourages a reasonable amount of infected cell death because it ultimately results in lessened viral load. Effectively, the present methods and compositions provide an alternative means to cytotoxic T cell responses in ridding the body of infected cells.

[0027] In yet anotehr aspect of the ivnention, the method for treating and/or inhibiting Human Immunodeficiency Virus (HIV) infection in an animal may be described as comprising: transfecting an autologous stem cell sample from a patient in need of treatment with the retroviral vector of claim 2 to provide a population of cells comprising transformed autologous cells that constituitively express a selected αHIV-Group 1 trans-splicing intron; and administering said transformed autologous stem cells to the animal to provide a treated animal, wherein transformed autologous stem cells and cells derived therefrom that become infected with Human Immunodeficiency virus in the treated animal will undergo apoptotic cell death. In some embodiments, the transformed autologous stem cells comprise reconstituted susceptible cells with a targeted trans-splicing molecule that generates apoptotic cell death in the presence of HIV mRNA. In the presence of the transformed population of stem cells, the ability of the HIV virus to re-establish infection in the treated animal is eliminated. In some embodiments, the animal is a human.

[0028] In some embodiments of the method, the patient/animal may also undergo a supplementary immune ablation treatment prior to receiving the transformed autologous stem cells. In other embodiments, the patient/animal may further be treated with a step of receiving a highly active retroviral treatment (HAART), such as to augment suppression of the HIV infection. The autologous stem cell sample from the patient may particularly comprises patient-derived bone marrow stem cells or hematopoietic stem cells.

[0029] In yet another aspect, an αHIV-Grpl intron construct is provided. In some embodiments, the construct may be defined as comprising a 3' apoptosis-inducing gene sequence; (b) an external guide sequence (EGS); (c) an internal guide sequence (IGS); and (d) a conserved HIV targeting sequence that corresponds to an HIV genome

Primer Binding Site (PBS), a Primer Activation Site (PAS), or a combination thereof. In some embodiments, the αHIV-Grpl intron construct may be defined as a PAS126 intron, a PAS128 intron, a PBS 182 intron, or a LOOP 126 intron.

Brief Description of the Drawings

[0030] **Figure 1a-1b**: Figure 1a - Group I intron structure and activity. Left: Structural features of Trans-splicing Group I introns showing alignment with the target sequence, the relative positions of the antisense IGS and EGS, as well as the P10 helix and intron catalytic core (Intron). **Figure 1b**: Trans-splicing reaction catalyzed by the group I intron. *First step:* Intron finds its target RNA sequence through complimentary base pairing with the guide sequences. The 3' GNP OH attacks the phosphodiester backbone directly downstream of the reactive uracil on the 5' exon. *Second step:* The 3' exon is brought into proximity with the newly freed 3'-OH on the cleavage uracil, guided by the P10 helix. The 3'-OH attacks the phosphodiester backbone just upstream of the 3' exon in another transesterification reaction, resulting in the 5' exon and the 3' exon being joined covalently. The end result is a new RNA molecule.

[0031] **Figure 2:** The 5' region of the HIV genome was amplified from a lentivirus vector, pLVX-Puro (Clonetech), inserted into the pBSK+downstream of the T7 promoter for in vitro expression as RNA.

[0032] **Figure 3:** Provirus sequence and transcription map showing the inclusion of the PBS at the 5' end of all transcripts produced from an integrated HIV provirus (Lamothe and Joshi (2000)).

[0033] **Figure 4:** $tRNA^{lys3}$ binds HIV-2 to prime reverse transcription. Binding interaction is highly conserved between HIV-1 and -2. In HIV-1, PAS is "GACCUGG" rather than HIV-2's "GACCCUGG" pictured. Initial bases added are those immediately upstream of the PBS. Figure adapted from Freund et al. (2001).

[0034] **Figure 5:** Schematic depicting the binding of the Loop126 anti-HIV group I introns to the conserved PBS and PAS sequences. The intron binds in a similar way as

the $tRNA^{lys3}$ binds to these sequences, allowing a large loop bulge which forms a natural stem loop structure. Such a large loop bulge region has previously never been designed into a trans-splicing Group I intron, and is one of the innovative aspects of the design of these introns. The fact that incorporation of such a large loop bulge does not significantly detract from the trans-splicing activity permits applications of these introns where conserved regions are separated by more than a few non-conserved sequences.

[0035] **Figure 6:** Sequencing results of RT-PCR recovered splice products from *in vitro* trans-splicing reactions with PBS/PAS targeting Group I introns.

[0036] **Figure 7:** Trans-splicing activity of αHIV-Grpl introns in transiently co-transfected 293 cells. A. Lentivirus expression plasmids were constructed to express the αHIV-Grpl introns from the CMV promoter. B. The target transcript for firefly luciferase was expressed from the native HIV-1 LTR promoter to generate transcripts having the 5' terminal sequences of native HIV, including the PBS and PAS targets. C. Target transcript, αHIV-Grpl introns, and splice products were detected in cells 96 hours post transfection using RT-PCR with sequence specific primers. The introns PAS 128 and PAS 128L (L stands for long external guide sequence) yielded the best results in this assay.

[0037] **Figure 8:** Cells were infected with the HIV-iGFP chimeric fluorescent virus, incubated for 48 hours, and then transfected with pLXRN CMV expression vectors expressing αHIV-Grpl intron 128L either with (128L tBax) or without (128L) an attached 3' tBax exon. Controls were HIV-iGFP infection alone (HIV) and post infection transfection with a DsRed expression plasmid (DsRed). Virus amounts are recorded as relative fluorescence in virus supermutants, and are relative to the HIV infection control.

Detailed Description of the Embodiments

[0038] Presented are αHIV-Grpl intron constructs that are useful in the control and inhibition of HIV infection. Methods employing these constructs are also provided that present a method for treating and/or curing HIV and the progression thereof the AIDS. Several assays for trans-splicing activity are provided that employ both artificial sequences and infectious targets. These designs involve the addition of an extended external guide

sequences (EGS) that functions to improve recognition, alteration of a p10 helix to provide an even more favorable splicing context, and lesser conserved targets within the same region of the HIV genome. A quantitative RT-PCR protocol is developed to make more quantitative comparisons of the data.

Example 1 – Construction of Retroviral Vectors

[0039] The present example demonstrates the construction and use of retroviral vectors containing αHIV-Grpl introns having a 3' apoptosis-inducing gene to establish transformed cells that constitutively express these selected Group I introns, and that may be used in the treatment and cure of a human immunodeficiency virus in an animal. These transformed cells are challenged with active HIV virus to demonstrate the efficacy of the introns in suppressing lentivirus infections in the transformed cell cultures. Retrovirus vectors will be constructed for transduction of these expressed introns in HEK293 cells.

[0040] Following selection and cell sorting, the effectiveness of these introns in suppressing HIV infection of these transformed cell cultures will be assessed.

Example 2 – Apoptosis-inducing gene products for Suppression of HIV Infection

[0041] The present example demonstrates the effectiveness of alternative apoptosis-inducing gene products for suppression of HIV infection in the present αHIV-Grpl intron strategy. Initial examinations into apoptosis inducing gene products focused on the tBax inducer. While the tBax protein has proven to be an effective inducer of apoptosis in both the mosquito cell and human cell applications, it does not have enzymatic activity, and may therefore be inferior to other inducers of apoptosis such as Caspases. The present invention will employ certain caspases as an alternative 3' exon for apoptotic induction following trans-splicing.

Example 3 – Hematopoietic Stem Cell Replacement

[0042] The present example presents a transgenic αHIV-Grpl intron hematopoietic stem cell replacement strategy in a mouse system. A chimeric HIV virus will be used that has an the envelope coding domain from an ecotropic MLV that restricts replication of the chimeric virus to rodents (Potash et al., 2005). This will be used to establish infections in young adult mice. These mice will serve as models for the present transduced hematopoietic stem cell therapies using the αHIV-Grpl introns.

[0043] While this proposal is designed to validate this approach using a less costly and more rapid mouse system, the data provided will serve to provide support for the successful expectation of the use of this approach in simian models.

[0044] This invention provides a means of eradicating HIV virus from an infected individual. This strategy will be effective either alone or in conjunction with other strategies currently being used and/or proposed for use for transduced hematopoietic stem cell therapies.

Example 4 – Studies targeting conserved sequences of the HIV Genome

[0045] Two sequences that are highly conserved within the HIV genome are the ψ-packaging signal (Amarasinghe et al., 2000) near the 5' end of the *gag* gene and the adjacent upstream $tRNA^{lys3}$ primer binding site (PBS). The accessibility of these conserved sequences to Group I intron attack was examined by constructing a 540 nt long target sequence encompassing the 5' terminus of the HIV genome and including the tRNA PBS and ψ-packaging signal (Fig. 5).

[0046] Group I introns targeting the ψ-packaging signal were unsuccessful because the targeted sequence for cleavage occurred in a complex of stem loop structure that was not accessible to the intron under normal physiological conditions.

[0047] The $tRNA^{lys}$ primer binding sequence was then examined. The $tRNA^{lys}$ primer binds the host genome at three sites: the PBS (Primer Binding Site), the PAS (Primer Activation Signal), and anticodon recognition sequence (Dobard et al., 2007).

Abbink et al., (2004) recognized that the PBS is almost completely conserved across HIV as it is encoded by the tRNA primer[19]. The PBS-tRNA interaction is stronger than the other two sites because of the greater number of base pairs. A point mutation in the PBS has been seen to occur if a virus uses $tRNA^{lys5}$ to prime reverse transcription instead, but frequently this mutation will revert by binding $tRNA^{lys3}$ imperfectly in the next generation's reverse transcription. The PAS motif also appears to be necessary to initiate reverse transcription and thus should also be well conserved.

[0048] A Quick Align search using the Los Alamos National Laboratory HIV sequence database reveals the PAS is fairly well conserved, though a few point mutations exist within several viral clades. The PBS is nearly perfectly conserved across the major clades B, C, and D. Clades B is the most prominent clade in the US and Western Europe, and displays a highly conserved PBS and PAS, while Clade D, prevalent in Eastern Europe and Sub-Saharan Africa, display 100% conserved PBS and PAS. In nearly all sequences the PBS's upstream flanking sequence is perfectly conserved as well. Unfortunately, the first several hundred nucleotides of the viral genome (including the presently described sequence of interest) are not well represented in Los Alamos' sequence database, and the smaller number of sequences observed could misrepresent the true conservation of the PAS and PBS. By both the conservation and accessibility, it was determined that the PAS and PBS would be good candidates for intron targeting.

Example 5 – Design and testing of αHIV-Grpl Introns

[0049] Targetable uracils were identified, two within the PAS (U126 and U128) and one within the PBS (U182). Four introns were then designed. Three standard introns targeted each uracil (PAS126, PAS128, PAS 182), and a fourth (LOOP126) targets U126 from the PAS and base pairs with the PBS via EGS. This latter intron is quite different from the previously designated introns, and novel among all published introns, as the region of non-homology termed the "loop bulge" in the target HIV RNA is a rather large stem loop that forms between the PAS and PBS sequences (Fig. 5). The intron models $tRNA^{lys3}$ in binding both PAS and PBS. Successful splicing was observed in our in vitro reaction system for each of the Group I introns targeting the PBS and PAS sequences (Fig. 6).

[0050] Splicing assays were also performed against target plasmids containing the 5' LTR sequence of HIV-1 in transiently transfected 293 cells. Each αHIV-Grpl intron was expressed from the CMV promoter of a lentiviral vector construct (Fig. 7b) in the presence of a co-transfected plasmid that expressed a firefly luciferase gene transcript from the HIV-1 LTR (see Fig. 7b). The resulting RT-PCR analysis (Fig. 7c) revealed the presence of spliced product, and subsequent analyses confirmed proper splicing of the 3' exon by the αHIV-Grpl introns.

Example 6 – Confirmation of αHIV-Grpl intron activity against HIV

[0051] While activity assays against the plasmid-encoded LTR-linked sequences demonstrated the splicing activity of the αHIV-Grpl introns, the present study was conducted to confirm that this activity would translate into an effective suppression of HIV in a productively infected cell.

[0052] A preliminary study was conducted using the αHIV-Grpl intron 128L. A previously developed GFP labeled HIV virus clone, HIV iGFP (Hubner et al., 2007), was employed. This clone that allows fluorescent quantification of virus production in 293 cells. Virus produced with this clone was used to establish infection in 293 cells for 48 hours, at

which time the infected cells were transfected with pLXRN CMV vectors (see Fig 7A) expressing αHIV-Grpl introns having either nonsense sequence or tBax as the 3' exon. After a further 96 hours, virus was collected from the supernatants and the EGFP fluorescence was quantified using a Spectramax M5e.

[0053] The results (Fig. 8) clearly show a significant reduction in the production of labeled virus in the presence of the αHIV-Grpl intron, whether or not tBax was used as the 3' exon. In the absence of tBax, reduction levels reflect the inhibition of new virus as a result of the activity of the ribozyme alone. In contrast, when tBax is substituted as the 3' exon, virus reduction is even greater.

[0054] Because these assays were done using transfected expression plasmids and established HIV infections in the cells, they may not accurately reflect the levels of reduction expected if cells are first transformed to express the αHIV-Grpl intron and then challenged with HIV. In fact, priming the cells with expressed αHIV-Grpl intron prior to infection with virus should lead to significantly better protection of the cultures and significantly greater reductions of produced virus.

Example 7 – HIV Treatment and Validation

[0055] The present example validates an αHIV-Grpl-apoptosis effector molecule for suppression of HIV in transgenic hematopoietic stem cell replacement therapies. The method is designed to ultimately test the efficacy of this approach in an animal model system. The model system of choice for these first analyses is the mouse. This strategy as validated in this less expensive, more easily analyzed model system will provide a solid foundation to propose later primate studies.

[0056] The present example presents the construction and evaluation of additional αHIV-Grpl introns utilizing the several previously established assays for trans-splicing activity employing both artificial sequences and infectious virus targets.

[0057] While the present initial results with constructing and testing αHIV-Grpl introns have been relatively productive in that one intron has been identified that seems to

have optimal activity, improvements in the activity of the other introns designed can be made with further design changes. These design changes involve the addition of extended external guide sequences (EGS), alterations of the p10 helix to provide a more favorable splicing context, and examination of less conserved targets within the same region of the genome. The present intron constructs also include an Internal Guide Sequence (IGS), whose sequence/structure may also be optimized for the present intron constructs.

[0058] Bell et al. (2004) suggest several ways to optimize the activity of Group I introns. For example, shortening the P10 helix and lengthening the P9.0 domain to eight base pairs can increase the efficiency of the second step reaction of trans-splicing.

[0059] *Study design:* An *in vitro,* transiently transfected cell system will be used, and HIV-iGFP assays will be utilized for determining the activity of each αHIV-Grpl intron. All assays will employ RT-PCR as a first analysis to confirm splicing activity. A quantitative RT-PCR protocol will be developed to make more quantitative comparisons of the data. Up until this point the data have been qualitative only with respect to the RT-PCR analyses, and even though there may appear to be differences in activities in the *in vitro* and transient transfection assays, these differences may reflect subtle differences in assay conditions or variability in concentrations of substrate and intron rather than true differences in activity of the introns.

[0060] Activities of all new constructs will be evaluated against the 128L standard as a control. While consistently good results were obtained with the 128L intron in the present assays, other introns may also be similarly effective if the assays are repeated and optimized. In addition, while this standard may represent the most effective intron under conditions of the assays, further examination of the introns may be pursued that appear to yield somewhat less effective results in the context of these particular assays in the event that acceptable results would still be obtained when applied in the context of the transformed cell assays as described in above.

[0061] Retroviral vectors containing αHIV-Grpl introns having 3' apoptosis-inducing genes will be constructed and used to establish transformed cells that constitutively express these selected Groups I introns. These transformed cells will be

challenged with active HIV virus to test the efficacy of the present introns in suppressing lentivirus infections in transformed cell cultures.

[0062] Thus far none of the assays employed to test the activity of the present αHIV-Grpl introns are reflective of the actual conditions under which the introns are expected to be employed in an HIV infection suppression scenario. For this reason, transformed cell cultures will be developed that constitutively express the αHIV-Grpl intron, and will then be exposed to low levels of infectious HIV virus.

[0063] Transformed 293 cells will be prepared that express the present αHIV-Grpl introns. Cultures of these transformed cells will then be used to determine the effectiveness of these introns in suppressing HIV infection. To do this, the retrovirus vectors will first be altered for transduction of the expressed introns in a way that permits optimized selection of the resulting transformed cells.

[0064] ***(a). Modification of the pLXRN-based vector to express the mCherry fluorescent marker gene***: The pLXRN-CMV base vector will be altered to incorporate an ECMV IRES directed mCherry downstream of the 3' exon of the αHIV-Grpl introns. The effectiveness of incorporating an IRES expressed downstream introns has already been demonstrated (Carter et al., 2010). The advantage of employing this intimately linked marker gene is two-fold. First, this will establish that if cells are expressing the mCherry, they are also expressing the αHIV-Grpl intron, since both are part of the same di-cistronic transcript. Second, the mCherry fluorescence provides a handle useful to sort the transformed cells for enrichment following hygromycin selection.

[0065] In previous analyses with the αDENV-Grpl introns, a level of background DEN virus infection was observed in hygromycin selected cultures. This level may be due to the presence of hygromycin resistant untransformed cells in the cultures because these levels are influenced by the stringency of selection and the freshness of the hygromycin. To insure optimal results, inclusion of a marker that can provide a handle for cell sorting may be used.

[0066] Finally, by validating this IRES-coupled gene expression approach with respect to the αHIV-Grpl introns, the introduction of a coupled selectable gene that may be

useful in subsequent development of *in vivo* applications is supported. Specifically, this will establish that it is possible to provide an IRES-expressed, intimately linked gene that under given conditions (e.g., drug exposure), would permit selective amplification of transformed hematopoietic stem cells in a patient without the need for the more extreme measure of first ablating the immune system.

[0067] ***(b.) Transformation of 293 cells with lentivirus vectors expressing the αHIV-Grpl introns and testing for HIV infection***. 293 cells will be infected with each lentivirus vectors at an MOI of 10 to insure relatively complete infection of the cell culture. Following a 72 hour recovery period, the cells will be subject to staged selection with hygromycin over a three week period, monitoring mCherry fluorescence to insure enrichment for those cells expressing the αHIV-Grpl intron. The expression of each intron will also be confirmed using qRT-PCR. Following the three week selection period we will sort the cell populations using a BD Biosciences FACSAria III cell sorter to enrich for optimal expression of the mCherry marker, which should also insure optimal expression of the αHIV-Grpl intron.

[0068] The negative control for trans-splicing activity will be produced by removing of domains P4 through P6 of the trans-splicing domain (Cech, 1990). Domains P4 to P6 must be removed instead of only the Pabc5 domain, as previously described (Ayre et al. 1999), since in our previous analyses αDENV-Grpl ΔPabc5 retained residual activity. Domains P4, P6 and Pabc5 form an extensive interface with each other to form the basis of the catalytic core (Murphy and Cech 1993; Murphy and Cech 1994; Cate et al. 1996).

[0069] ***(c.) Challenge of αHIV-Grpl intron expressing cells with HIV***: The HIV-iGFP virus (Hubner et al., 2007) will be used as well as wild type NL4-3 in assays for the effectiveness of HIV suppression in our transformed cell cultures. While the HIV-iGFP mutant virus has the advantages of ease of assaying, there are some problems with relative infectivity compared with the wild type virus (Muller et al., 2004). In addition, the fluorescent counts alone cannot be considered reliable predictors of actual infectious virus production. Therefore, the standardized p24 assays will be used as well as qRT-PCR assays to determine the relative effectiveness of our αHIV-Grpl introns in suppression of

virus infection of the transformed cells. Finally, the effectiveness of the apoptotic response will be assessed using Annexin V staining to confirm that apoptotic cell death is occurring in HIV-challenged cultures.

[0070] ***iii.) Examining the relative effectiveness of alternative apoptosis-inducing gene products for suppression of HIV infection in our αHIV-Grpl intron strategy.***

[0071] While the tBax protein has proven to be an effective inducer of apoptosis in both the mosquito cell and human cell applications, it does not have enzymatic activity that could effectively enhance its performance. The buildup of sufficient tBax is relied upon as a result of the splicing and translation reaction, and as a result this may not be the a somewhat less effective means of inducing apoptosis than generating a protein with enzymatic properties.

[0072] It may be more advantageous to use caspases as a 3' exon since it takes multiple tBax molecules to form a single pore, while the enzymatic activity of the caspase amplified its effect within the cell. Listed below are several caspases that may be of interest.

[0073] *Caspase 3*: Activiated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways making this the most logical candidate as the expression of the active form of this caspase would activate both pathways simultaneously (Salvesen 2002; Ghavami et al., 2009), leading to activation of other caspases that will cleave of cellular substrates and resulting in apoptosis.

[0074] *Caspase-6*: Plays a central role in the execution phase of apoptosis activating targets following activation by initiator caspases (Cowling and Downward, 2002). Activation of caspase 6 in the absence of initiator caspases in cells would lead to the initiation of apoptosis without down regulation of this event by inhibitors of apoptosis (IAP) that act upon initiator caspases.

[0075] *Caspase 8:* An initiator caspase. According to the "induced-proximity model" (Salvensen and Dixit, 1999) procaspase-8 undergoes autoproteolytic cleavage, following recruitment to the death-inducing signaling complex (DISC) forming active

caspase-8, which in turn can activate other procaspases, leading to cleavage of cellular substrates, and apoptosis.

[0076] *Caspase-9*: Activated Caspase-9 is able to cleave Caspase-3(Li et al., 1997) leading to initiation of the extrinsic and intrinsic apoptotic pathways.

[0077] Each of these caspases exists in an inactive and an active form. In some cases modification of the sequence can lead to a constitutively active enzyme that, when expressed, will irreversibly induce apoptosis (Srinivasula et al., 1998). These sequences may be designed to serve as 3' exons similarly to the way we designed the tBax 3' exon.

[0078] Sequences encoding the active forms of each caspase will be placed in the 3' exon position of the 128L αHIV-Grpl intron for comparative analysis of their effectiveness in inducing apoptotic cell death in transformed 293 cells challenged with HIV. The standard assays for splicing and apoptosis induction will be performed as above.

[0079] ***iv.) Modeling a transgenic αHIV-Grpl intron hematopoietic stem cell replacement strategy in a mouse system.***

[0080] Mouse models for HIV infection are limited in their relevance, particularly when considering immunological responses. Even humanized mouse models fall short of predictive validity in this respect. However, the approach here examined does not rely on immune function for its effectiveness. In fact, immune function is irrelevant to the characterization of the effectiveness of the present αHIV-Grpl intron as a suppressive tool. Therefore, an appropriate mouse model could provide a cost effective and rapid means for obtaining valuable information justifying this transgenic hematopoietic stem cell replacement strategy.

[0081] Instead of relying on the more complex humanized mouse models, a chimeric HIV virus, Eco-HIV will be utilized that is built upon the clade B NL4-3 backbone. This virus has a replacement of the HIV gp120 sequence with the envelope coding domain from an ecotropic MLV that restricts replication of the chimeric virus to rodents (Potash et al., 2005). Infection of mice with this virus produces the repertoire of infected cell types typical of an HIV infection. Any mouse strain of choice may be inoculated with this chimeric virus and results in infection of all major target cell types of

HIV-1. Virus burdens in the spleen are comparable to HIV-1 burdens in resting lymphocytes in human (Potash et al., 2005). Therefore, this chimeric virus in the mouse system seems to be good choice for the present studies.

[0082] The chimeric virus will be used to establish infections in young adult (8-10 weeks) mice for a six week period. To insure complete infection we will sample hematopoietic tissues, particularly lymphocyte and macrophage populations, for the Eco-HIV virus. Assays will include circulating virus assays as well as PCR assays of provirus. Once infection has been established mice will be irradiated and/or subjected to chemical myeloablation followed by transfusion through tail vein with αHIV-Grpl intron transformed syngeneic hematopoietic stem cells. The HIV infection will be held in check with drug therapies to allow reconstitution of the immune system within the mice in the absence of significant virus load. Lymphocyte and macrophage cell populations will be monitored to insure full reconstitution. This strategy models clinical protocols for hematopoietic stem cell therapies (e.g. Cartier et al., 2009). Following the reconstitution of the immune system the antiviral therapy will be removed and the levels of lymphocyte and macrophage cell populations will be monitored to determine the impact of the active infection on these transformed cells. It is expected that there will be a reduction in the cell counts for HIV susceptible cell populations as a result of the impact of the DUI activity. The virus and provirus load will also be monitored to establish maintenance of a low viremia.

[0083] A unique and revolutionary strategy for combating and curing HIV infections is provided with the present anti-HIV Group 1 intron strategies. By providing reconstituted susceptible cells with a targeted trans-splicing molecule that generates apoptotic cell death in the presence of the HIV mRNA substrate following infection, the ability of the virus to re-establish infection in the transduced reconstituted immune cells is eliminated. This has the dual effect of stabilizing the viral load at sub-clinical levels, as well as acting as a "sponge" for virus shed from long term reservoirs. The key to this approach is successful attack of invariant sequence within the HIV genome, which have been identified and confirmed are subject to trans-splicing. Another advantage of this approach is the ability to couple the trans-splicing intron to an IRES-expressed gene product with

allows for engineering of a selective genes that insures maintenance of the transgene in reconstituted tissues, and may also allow repopulation of the immune system without myeloablation therapies.

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What is claimed is:

1. An antiviral molecule for use in the treatment of Human Immunodeficiency Virus infection in an animal, said antiviral molecule comprising an αHIV-Group 1 trans-splicing intron having a 3' exon encoding an apoptosis-inducing gene sequence or a caspace sequence, a sequence having binding affinity for a $tRNA^{lys}$ primer binder sequence (PBS), an Internal Guide Sequence (IGS) and an External Guide Sequence (EGS).

2. The antiviral molecule of claim 1 wherein the $tRNA^{lys}$ primer binder sequence is a primer binding site (PBS) sequence.

3. The antiviral molecule of claim 2 wherein the intron targets a uracil target site at uracil position at U126, U128, U182, or a combination thereof.

4. A retroviral vector comprising the antiviral molecule of claim 1, said retroviral vector being capable of transforming a population of cells that constitutively express the αHIV-Group 1 trans-splicing intron .

5. The retroviral vector of claim 4 comprising a lentiviral vector.

6. The retroviral of claim 5 further defined as a pLXRN-CMV based vector.

7. A method for treating and/or inhibiting Human Immunodeficiency Virus (HIV) infection in an animal comprising:

transfecting an autologous stem cell sample from a patient in need of treatment with the retroviral vector of claim 2 to provide a population of cells comprising

transformed autologous cells that constituitively express a selected αHIV-Group 1 trans-splicing intron; and

administering said transformed autologous stem cells to the animal to provide a treated animal,

wherein transformed autologous stem cells and cells derived therefrom that become infected with Human Immunodeficiency virus in the treated animal will undergo apoptotic cell death.

8. The method of claim 7 wherein the transformed autologous stem cells comprise reconstituted susceptible cells with a targeted trans-splicing molecule that generates apoptotic cell death in the presence of HIV mRNA.

9. The method of claim 7 wherein the ability of the HIV virus to re-establish infection in the treated animal is eliminated.

10. The method of claim 7 wherein the animal is a human.

11. The method of claim 7 comprising performing an immune ablation treatment to the pateint prior to administering said transformed autologous stem cells.

12. The method of claim 7 further comprising the step of providing a highly active retroviral treatment (HAART) to the treated animal to augment suppression of the HIV infection.

12. The method of claim 7 wherein the autologous stem cell sample from the patient comprises patient-derived bone marrow stem cells or hematopoietic stem cells.

13. An αHIV-Grpl intron construct comprising:

(a) a 3' apoptosis-inducing gene sequence;

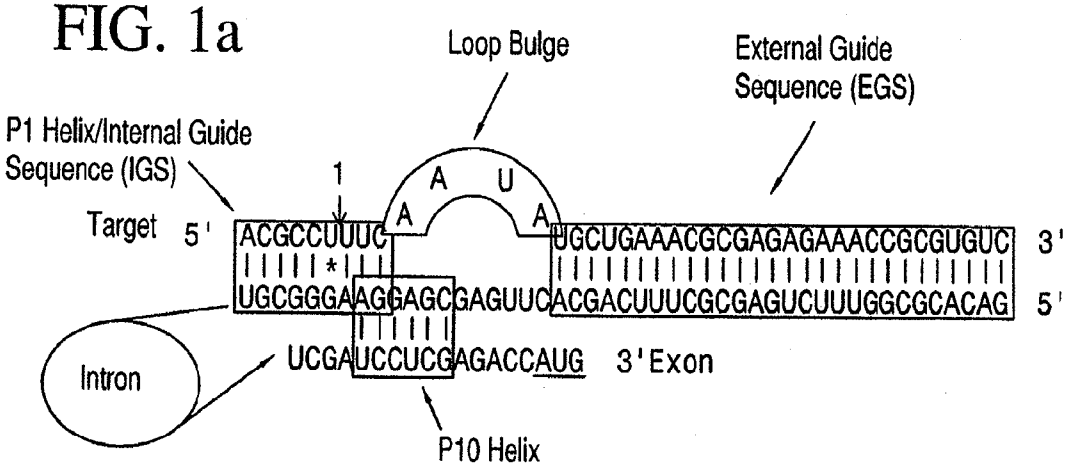
(b) an external guide sequence (EGS);

(c) an internal guide sequence (IGS); and

(d) a conserved HIV targeting sequence that corresponds to an HIV genome Primer Binding Site (PBS), a Primer Activation Site (PAS), or a combination thereof.

14. The αHIV-Grpl intron construct of claim 13 defined as a PAS126 intron, a PAS128 intron, a PBS 182 intron, or a LOOP 126 intron.

FIG. 1a


Loop Bulge
External Guide Sequence (EGS)
P1 Helix/Internal Guide Sequence (IGS)
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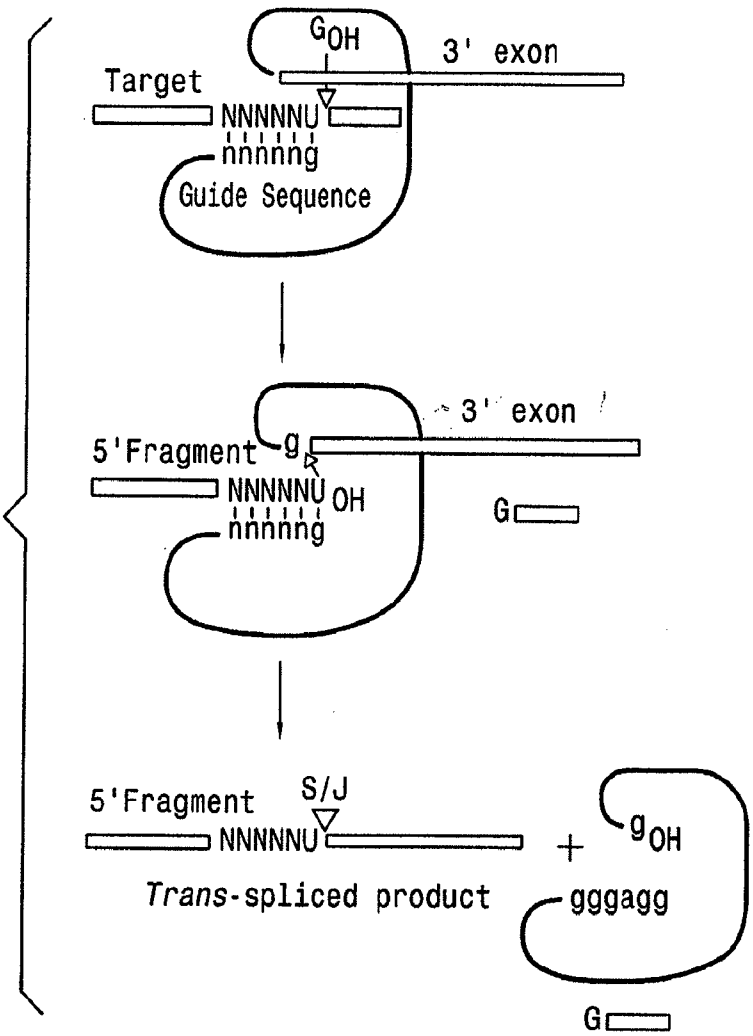
A U
A A
Target 5'
ACGCCUUUC
UGCUGAAACGCGAGAGAAACCGCGUGUC 3'
UGCGGGAAGGAGCGAGUUCACGACUUUCGCGAGUCUUUGGCGCACAG 5'
Intron
UCGAUCCUCGAGACCAUG 3'Exon
P10 Helix

FIG. 1b


GOH
3' exon
Target
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nnnnng
Guide Sequence
3' exon
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S/J
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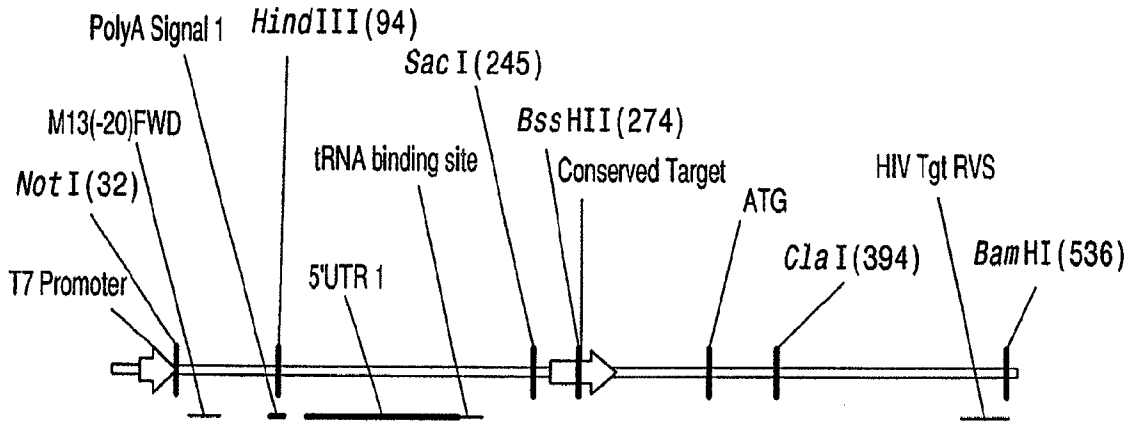
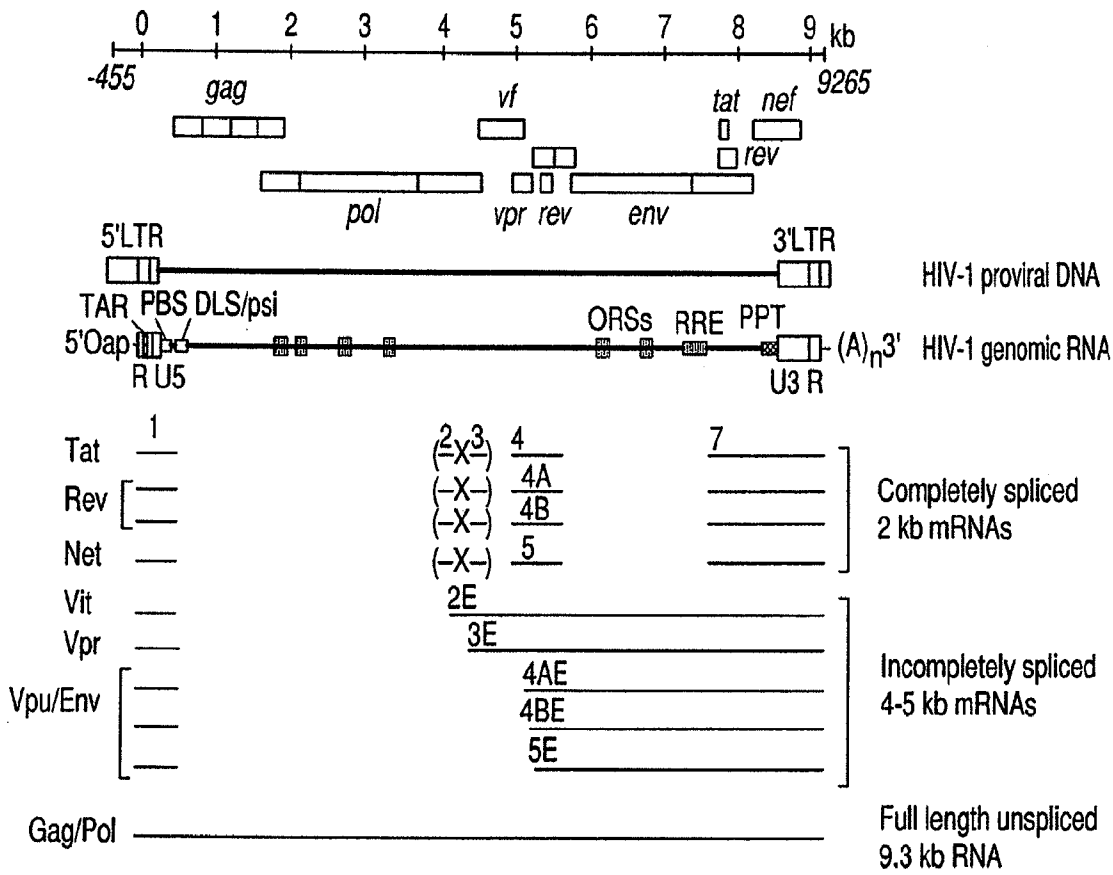
+
gOH
Trans-spliced product
gggagg
G



HIV-1 Target Sequence

540 bp

FIG. 2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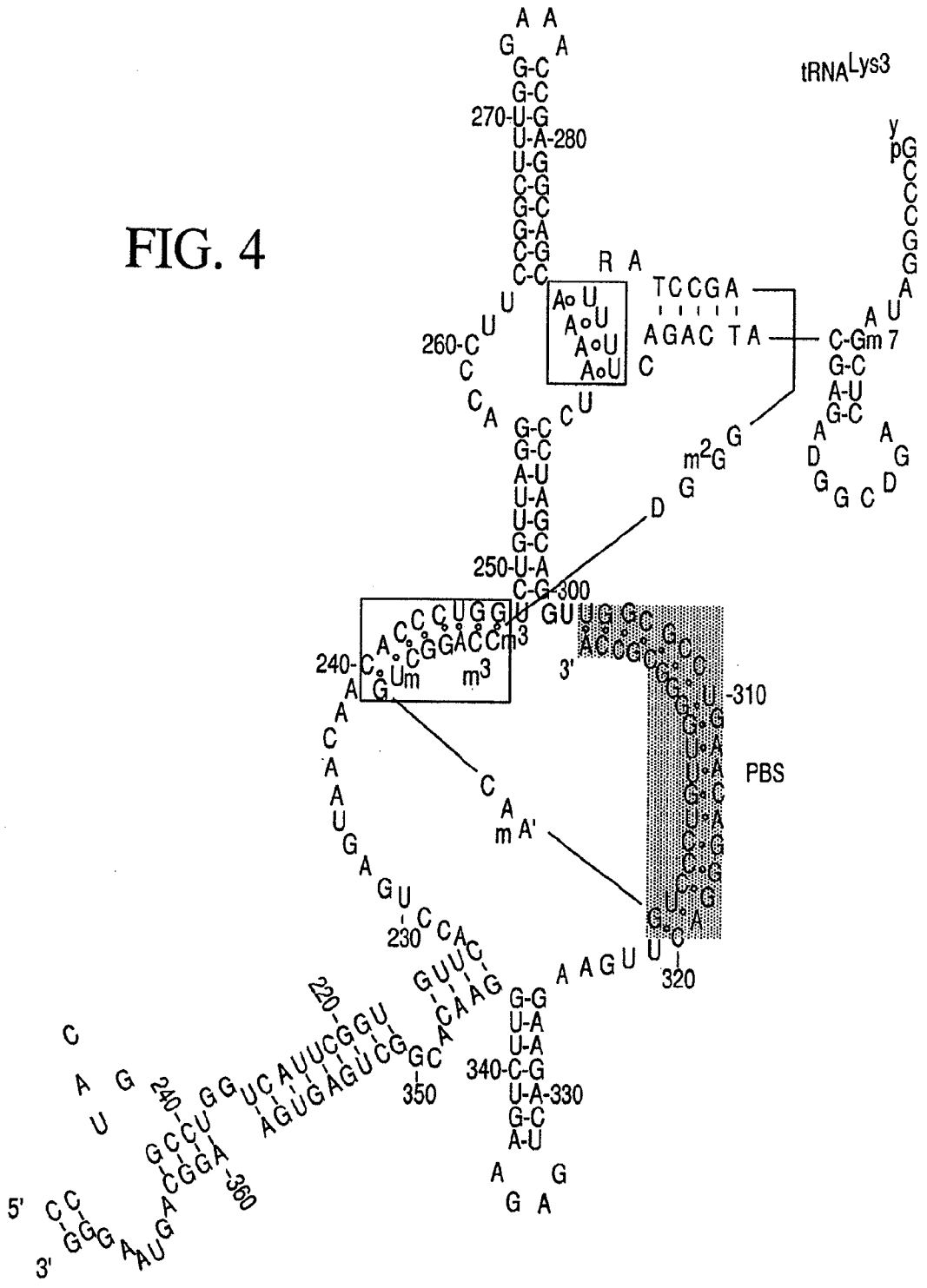
-455
9265
gag
vf
tat nef
rev
pol
vpr rev env
5'LTR
3'LTR
HIV-1 proviral DNA
TAR PBS DLS/psi
5'Oap
R U5
ORSs RRE PPT
(A)n3'
U3 R
HIV-1 genomic RNA
Tat
Rev
Net
Vit
Vpr
Vpu/Env
Gag/Pol
1
2 3
4
4A
4B
5
7
2E
3E
4AE
4BE
5E
Completely spliced
2 kb mRNAs
Incompletely spliced
4-5 kb mRNAs
Full length unspliced
9.3 kb RNA

FIG. 3

FIG. 4



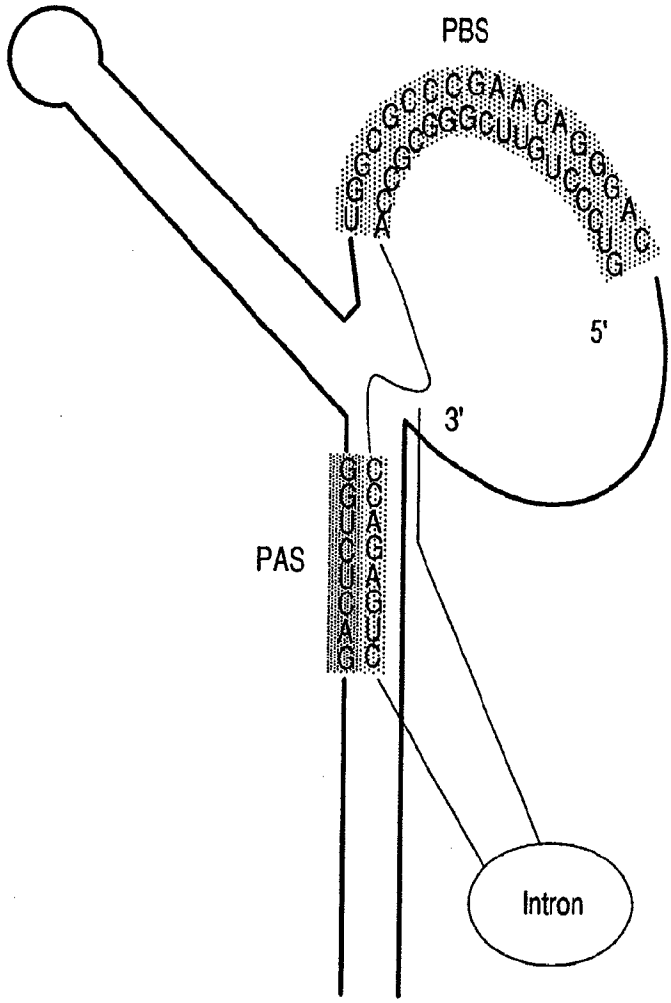


FIG. 5

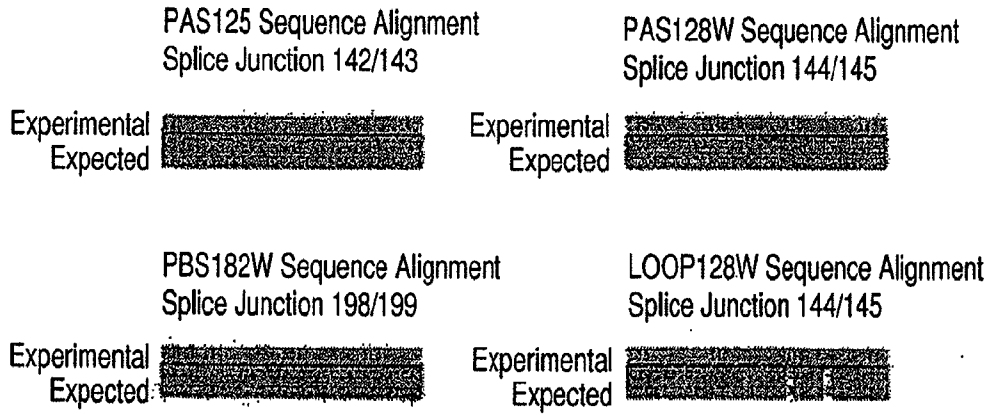

PAS125 Sequence Alignment
Splice Junction 142/143
Experimental
Expected
PAS128W Sequence Alignment
Splice Junction 144/145
Experimental
Expected
PBS182W Sequence Alignment
Splice Junction 198/199
Experimental
Expected
LOOP128W Sequence Alignment
Splice Junction 144/145
Experimental
Expected

FIG. 6

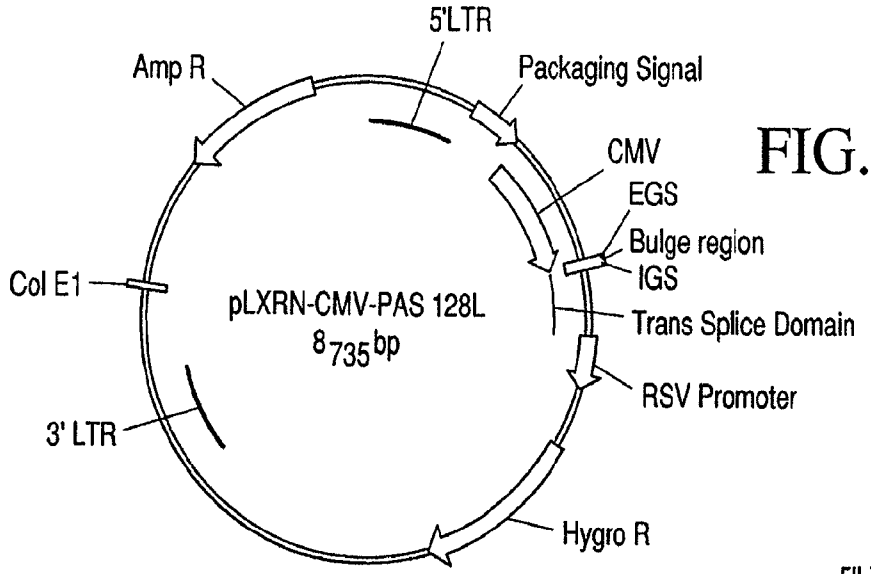


FIG. 7A

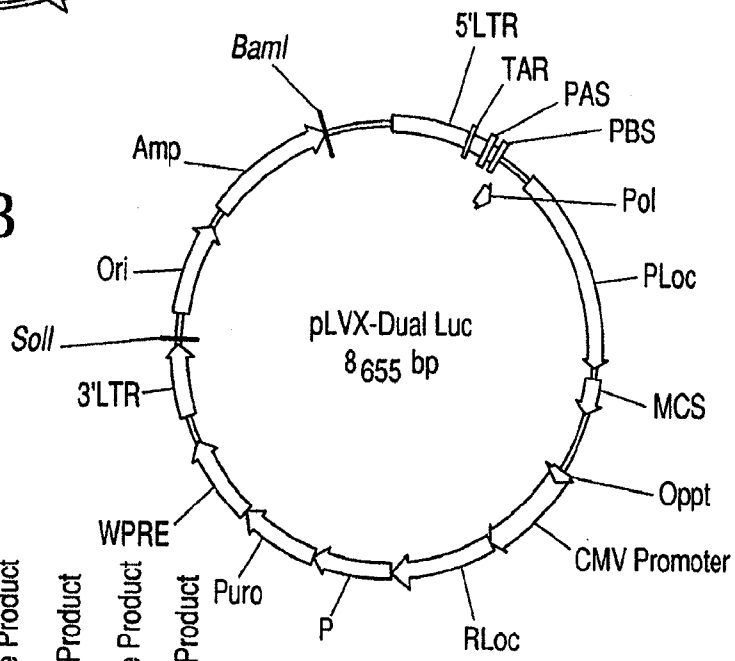
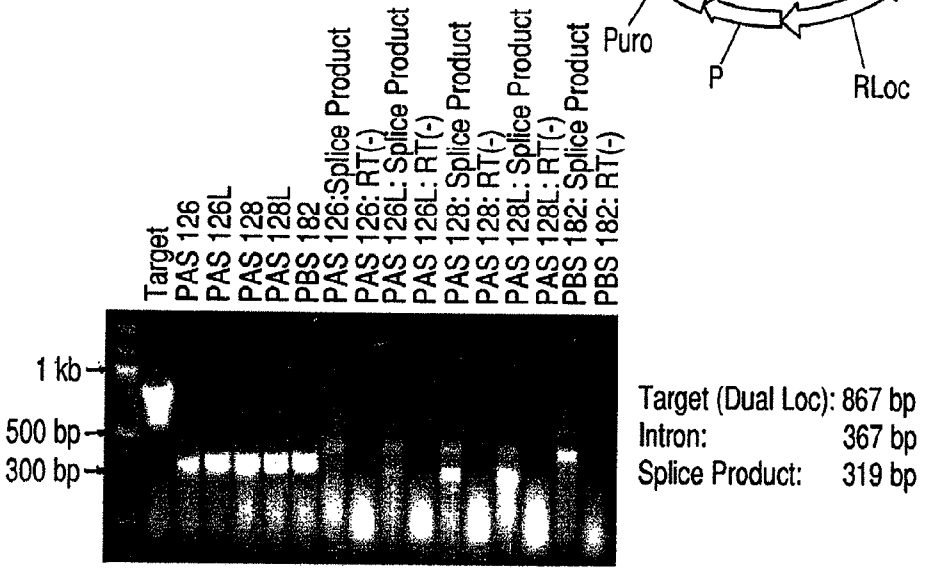


FIG. 7B

FIG. 7C



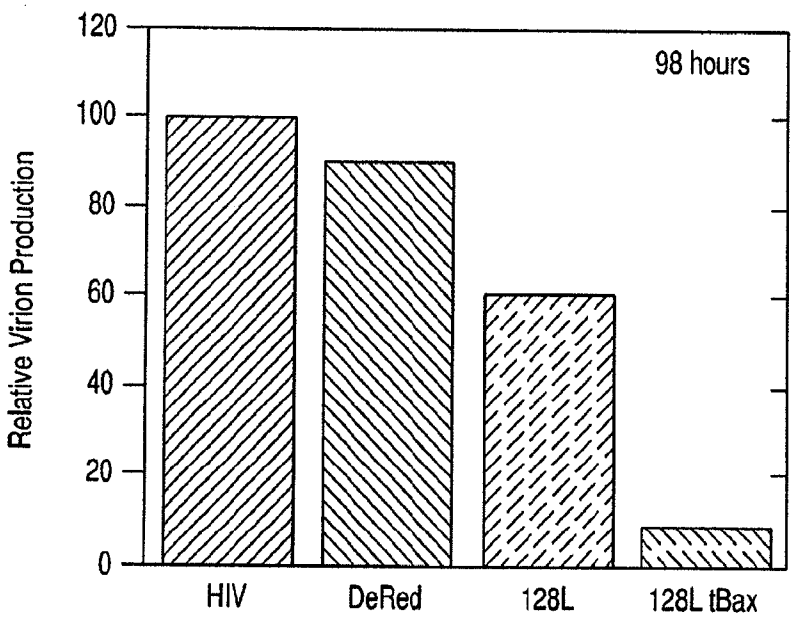

120
100
80
60
40
20
0
Relative Virion Production
98 hours
HIV
DeRed
128L
128L tBax

FIG. 8