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HUMAN IMMUNODEFICIENCY VIRUS TYPE I AS A TARGET FOR GENE THERAPY

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1. ABSTRACT

Recent progress in our understanding of the human immunodeficiency virus type 1 (HIV-1) life cycle has lead to the identification and characterization of viral genes or gene products that have been evaluated as targets for gene therapy. Virtually every stage in the viral life cycle and every viral gene product is a potential target. Gene therapy approaches directed at several of these viral targets have been successful at inhibiting HIV-1 replication in cultured human cells, but clinical trials involving gene therapy directed at HIV-1 are still in their infancy. This manuscript begins with a brief review of the viral life cycle with an emphasis on the function of viral gene products and then summarizes the gene therapy approaches that have targeted these viral genes or gene products to inhibit HIV-1 replication.

2. INTRODUCTION

Since the first cases of acquired immunodeficiency syndrome (AIDS) were reported in 1981, over 20 million people world-wide have become infected with human immunodeficiency virus type 1 (HIV-1), the etiologic agent for AIDS (1). Because of the large world-wide impact of AIDS and because of the speed with which the AIDS pandemic has spread, there has been an intense global effort into understanding the biology of HIV-1 and the host response to HIV-1 infection. This effort has resulted in rapid progress toward understanding HIV-1 pathogenesis, including an improved understanding of viral dynamics and the viral life cycle. These advances have lead to the development of several new drugs that are effective against HIV-1. Because of the rapid mutation rate of HIV-1, viral resistance to these new drugs is a major problem. Therefore combinations of drugs have been used to treat patients with HIV-1 infection with the goal of completely suppressing viral replication so that drug resistance mutations cannot occur. Although new drug combinations can suppress plasma RNA concentrations to low or undetectable levels in a large proportion of patients (2, 3), not all patients respond well to therapy and drug resistance is still a problem. In patients that do respond, the duration of the response is unknown, and the side effects related to long term use of antiretroviral combinations is also unknown. In addition, these new combination drug therapies are very expensive, thus limiting their use in less developed countries. Because of these problems, there is a need for continued research on the pathogenesis and treatment of HIV-1 infection.

In addition to developing new drugs that are effective against HIV-1, considerable research effort has been directed toward blocking HIV-1 using gene therapy approaches. Advances in our understanding of the viral life cycle has lead to the identification of numerous potential viral targets for gene therapy. Gene therapy approaches directed at several of these viral targets have been successful at inhibiting HIV-1 replication in cultured human cells. These promising results in cell culture

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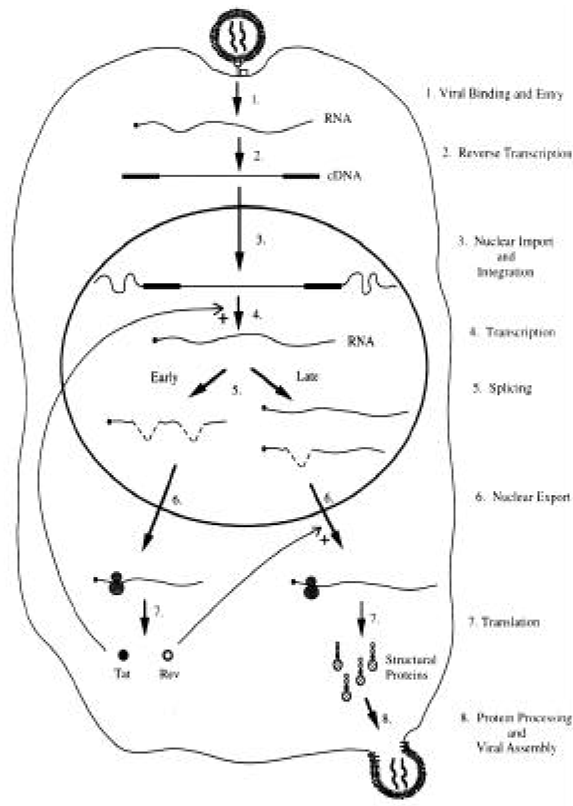

Figure 1. The HIV-1 life cycle.

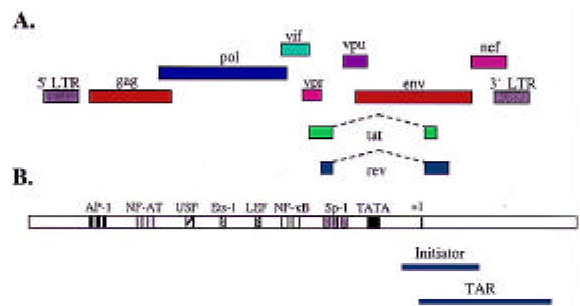

Figure 2. The HIV-1 genome. A. A schematic representation of the HIV-1 gene products encoded by the HIV-1 genomic sequence. B. A schematic representation of the HIV-1 promoter which is located within the 5' LTR of the viral genome. The core promoter consists of the initiator region, the TATA box, and three Sp1 sites. An upstream enhancer contains binding site for several cellular transcription factors including NF-κB, LEF, Ets-1, USF, NF-AT, and AP-1.

experiments have lead to optimism about the future prospect of using gene therapy to treat HIV-1 infection. Several obstacles need to be overcome, however, including development of appropriate gene delivery systems and evaluating the safety of gene therapy approaches in humans. Clinical trials involving gene therapy directed at HIV-1 have begun but are still in their infancy. This manuscript begins with a brief review of the viral life cycle with an emphasis on the function of viral gene products and then summarizes the gene therapy approaches that have targeted these viral genes or gene products to inhibit HIV-1 replication.

3. HIV-1 LIFE CYCLE

It has recently become apparent that even during asymtomatic HIV-1 infection viral replication occurs at a very rapid rate. High-level virus replication occurs primarily in the lymph nodes, producing over 1-10 billion virions every day and destroying a similar number of T cells in the process (4, 5). A detailed understanding of the viral replication cycle (figure 1) and the functions of the viral gene products (figure 2) is needed in order to design effective antiviral strategies.

A schematic presentation of the replicative cycle of HIV-1 is shown in figure 1. The replicative cycle starts by binding of the virus envelope glycoprotein gp120 to CD4 on the cell surface (6-8). The viral envelope protein gp41 then fuses with the cell membrane, a process which requires a cellular coreceptor. Recent studies have identified the chemokine receptor molecules CCR-5 (9-13) and CXCR-4 (14, 15) as the major coreceptors required for entry of HIV-1. Other coreceptors have been identified (10, 12), which may also play roles in HIV-1 infection. Macrophage-tropic strains of HIV-1, which constitute the vast majority of virus present in newly infected individuals seem to use CCR-5, while T-cell tropic strains which generally appear late in the course of infection use CXCR-4 (16-18).

After fusion of the viral envelope with the cell membrane, the viral core enters the cytoplasm where viral RNA is immediately transcribed by the viral enzyme reverse transcriptase (RT) into double-stranded cDNA. RT has three activities essential for retrovirus replication; it is an RNA-dependent DNA polymerase (i.e. reverse transcriptase), but also has DNA-dependent DNA polymerase activity (for synthesis of the second strand of the proviral DNA) and an RNase H activity. After synthesis of the viral cDNA, a nucleoprotein complex (or preintegration complex) containing the viral cDNA, matrix protein, integrase and other viral proteins, is translocated to the cell nucleus through connection with the cell nuclear import pathway (19). The Vpr accessory protein mediates transfer of the preintegration complex to the cell nucleus (19), making it possible for HIV to infect nondividing cells (20-22). The proviral DNA is then integrated into the host genome into regions of active transcription by the action of viral integrase (23). The integration process requires a host chromosomal protein in addition to the viral integrase (24).

The integrated provirus is flanked by long terminal repeats (LTRs) at each 5' and 3' end of the genome (figure 2A). The LTRs consist of repeated segments which are termed U3, R and U5. The LTRs contain the promoter sequences and the transcript polyadenylation signal (25). The viral genome contains structural and regulatory genes that are necessary for viral replication. The HIV-1 provirus uses the host cell's transcription machinery to produce the RNA transcripts necessary for viral replication. HIV-1 transcription is regulated in large part by the viral promoter which is located in the 5' long terminal repeat of the viral genome (see figure 2B). Host cellular RNA polymerase II transcription complexes are recruited to the HIV-1 promoter by specific interactions with promoter sequences. The HIV-1 promoter is similar to endogenous cellular promoters found within host cells in that it contains a canonical RNA polymerase II TATA box as well as

Figure 3. The HIV-1 TAR sequence and structure.

Figure 4. A model for regulation of HIV-1 transcription by Tat and TAR. RNA polymerase II transcription complexes are recruited to the HIV-1 promoter (top panel). These complexes then begin transcripton of TAR RNA (middle panel). The newly synthesized TAR RNA interacts with Tat and cellular factors (bottom panel) and this interaction activates the transcription complex such that it becomes more elongation competent.

binding sites for cellular transcription factors (for a review see reference 26). In addition, the HIV-1 promoter contains a non-conventional initiator element that appears to be unique to HIV-1 but is similar to cellular initiator elements (26, 27). The core promoter consists of the TATA box, initiator element, and three tandem binding sites for the constitutively expressed cellular transcription factor Sp1, and all of the elements necessary for transcription initiation *in vitro* are found in this core region (27). An upstream enhancer contains two binding sites for the inducible cellular transcription factor NF-κB and binding sites for a number of other cellular transcription factors including LEF, Ets-1, USF, NF-AT, and AP-1 (26, 27) .

Although the cellular transcription machinery can sustain a low basal level of HIV-1 transcription, the viral protein Tat is required for high level HIV-1 transcription and activates HIV-1 transcription by greater than 100-fold (28-30). In the absence of Tat, most of the RNA transcripts produced from the HIV-1 promoter are short transcripts resulting from early transcription termination, whereas in the presence of Tat, the number of long full-length HIV-1 transcripts increases (31-34). Several cellular proteins have been shown to bind to Tat *in vitro*, including several components of the transcription machinery (32, 35-39). Tat has also been shown to bind to the RNA polymerase II holoenzyme (40, 41). The functional significance of these interactions is not clear, but it is possible that transcription complexes that contain Tat are capable of elongation competence, whereas transcription complexes lacking Tat produce predominantly short transcripts. Activation of HIV-1 transcription by Tat depends on an intact *trans*-activation response (TAR) RNA element. TAR is a 59 nucleotide RNA stem-loop structure that forms the 5' end of all HIV-1 transcripts (see figure 3). The TAR structure consists of two stems, a four nucleotide bulge, and a six nucleotide loop (42-46). Tat binds to the TAR bulge region *in vitro* (47-49) and may bind to the same region *in vivo* since mutations in the TAR bulge that inhibit Tat binding *in vitro* also inhibit Tat-mediated transcriptional activation *in vivo* (30, 50, 51). Interestingly, mutations in the TAR loop that have little or no effect on Tat binding *in vitro* abolish Tat-mediated transcriptional activation *in vivo* (52-55), suggesting that a cellular factor that recognizes the TAR loop is essential for Tat to activate HIV-1 transcription. Although the mechanism by which TAR RNA regulates Tat-mediated activation of HIV-1 transcription is still largely unknown, it has been suggested that RNA polymerase II transcription complexes that contain Tat bind to newly synthesized TAR RNA as it forms, and this binding activates the transcription complexes such that they become more elongation competent (56). This activation of RNA polymerase II transcription complexes may be mediated by a Tat-associated kinase which phosphorylates components of the transcription machinery (37, 57-61). A model for the regulation of HIV-1 transcription by Tat and TAR is depicted in figure 4.

Early on in the viral replicative cycle, newly transcribed mRNA is spliced multiple times by the cellular splicing machinery. The Tat, Rev and Nef proteins are produced from these multiply spliced transcripts and subsequently start to accumulate. Tat activates transcription as described above and the Rev protein regulates transport of HIV-1 transcripts from the nucleus to the cytoplasm. Rev is a 116 amino-acid protein which localizes to the nuclei and nucleoli of infected cells (62). Rev binds to a major groove in the Rev-response element (RRE), a 210 nucleotide RNA sequence found in HIV-1 transcripts (63, 64). As Rev levels reach a critical level of expression, a progressive shift in viral mRNA production from multiply spliced (encoding Tat, Rev and Nef) to singly spliced and unspliced transcripts occurs (62). The Rev protein is required for transport of singly spliced and

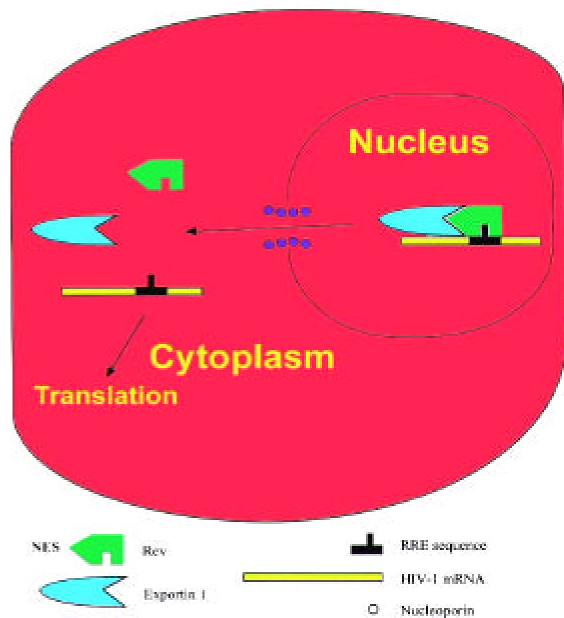

Figure 5. Rev-mediated HIV-1 mRNA export. Rev binds to a loop in the RRE sequence. Exportin 1, a cellular export factor, recognizes the nuclear export signal (NES) on Rev and the viral mRNA is subsequently transported by this complex from the cell nucleus through the nuclear pore to the cytoplasm where translation into proteins occurs. In addition to exportin 1, other cellular factors probably participate in this process.

unspliced viral mRNA from the nucleus to the cytoplasm where these transcripts are subsequently used to synthesize structural proteins and viral enzymes (62, 65). A nuclear export signal (NES) has been identified in the Rev protein (66, 67). Presumably, the Rev-NES recognizes and binds to the nuclear export factor exportin 1 (68, 69, reviewed in 70) which subsequently shuttles the Rev-mRNA complex from the nucleus to the cytoplasm (figure 5). Rev-dependent transport of RRE-containing mRNA also involves other cellular factors, including the Rev/Rex activation domain-binding (Rab) protein (71) and possibly the Rev-interacting protein (Rip1p), a small nucleoporin-like protein (72, 73). Presumably, the interaction between Rev and these cellular factors directs the transport of RRE-containing transcripts to the nuclear pore (figure 5).

The Nef protein is important for maintenance of high viral loads and for the development of AIDS (74). HIV infection leads to downregulation of surface expression of major histocompatibility complex class I (MHC-I) antigens (75, 76) and CD4 (77, 78) by endocytosis of these molecules from the cell surface. It has recently been shown that these effects are mediated by the Nef protein (78, 79). The stimulation of endocytosis by Nef could therefore represent a viral mechanism for evading the immune response because downregulation of MHC-I molecules makes HIV-infected cells less susceptible to lysis by cytotoxic T lymphocytes (75, 76, 80).

Once in the cytoplasm the unspliced viral mRNA is translated into a Gag-Pol polyprotein by a process that involves ribosomal frame shifting. The Gag-Pol polyprotein is cleaved by the viral protease into the mature virion structural proteins (matrix, capsid, and nucleocapsid) as well as the virion enzymes (protease, reverse transcriptase and integrase) (81). The viral envelope proteins are synthesized as the gp160 precursor polyprotein which is subsequently cleaved by cellular proteases into the external surface protein gp120 and the transmembrane envelope protein gp41. Viral assembly seems to be regulated at least in part by the accessory proteins Vpu and Vif (82, 83). Efficient packaging of genomic RNA of retroviruses into viral particles requires a specific nucleotide sequence, termed the ψ (psi) packaging sequence (84, 85). Packaging of HIV-1 RNA into virus particles then takes place, a process which involves an interaction between ψ and the zinc binding domains of the nucleocapsid protein (23, 86). Subsequently new virions are released from the surface of the host cell by budding and a new cycle can start.

4. TARGETS FOR GENE THERAPY

Increases in our understanding of the HIV-1 life cycle has lead to the identification of numerous potential targets for gene therapy. Of these potential targets, the interaction between Tat and TAR and the interaction between Rev and RRE have been studied the most extensively, although other viral targets have also been evaluated. This section summarizes the research that has been done to characterize viral molecules as targets for gene therapy.

4.1. Tat and TAR

Because the Tat/TAR interaction is essential for activation of HIV-1 transcription, several gene therapy approaches that target either Tat or TAR have been evaluated. Analysis of the effect of mutant Tat polypeptides on HIV-1 transcriptional activation has lead to the identification of *trans*-dominant negative Tat mutants that suppress HIV-1 gene expression (87-90) *Trans*-dominant negative Tat mutants likely function by blocking an essential interaction between the wild-type Tat/TAR complex and cellular cofactor(s). They may do this by binding to wild-type Tat, TAR or essential cellular cofactors. A subset of functional *trans*-dominant negative Tat polypeptides (87, 88) contain mutations in an activation domain that is necessary for Tat function and appears to bind to a cellular cofactor (91, 92). Over-expression of a Tat polypeptide with a mutation in this region was shown to inhibit activation from viral latency and to inhibit viral replication in a human cell line (87). Other *trans*-dominant negative Tat polypeptides that suppress HIV-1 gene expression (89, 90, 93) contain mutations in the basic domain of Tat which is involved in TAR-binding (49, 94). A potential problem with overexpressing *trans*-dominant Tat mutants as an approach to gene therapy is that these mutants may bind to and sequester important cellular factors and thereby disrupt normal cellular functions.

Intracellular immunization is another approach to gene therapy that has been applied to inhibiting the function of the Tat protein. Expression of a single chain intracellular antibody (or intrabody) against Tat effectively inhibited HIV-1 replication in stably transfected cells (95). This intrabody appeared to function by binding to and sequestering Tat in the cytoplasm, thereby preventing it from regulating HIV-1 transcription in the nucleus (95).

Gene therapy strategies that target *tat* or TAR RNA have also been studied. For example, expression of anti-sense *tat* RNA (96, 97) or expression of ribozymes designed to cleave *tat* RNA (97, 98) have been shown to inhibit HIV-1 replication in T cells lines. In one study, it appeared that expression of anti-sense *tat* RNA was

superior to an anti-*tat* ribozyme for inhibiting viral replication in a T cell line (97). Expression of anti-sense RNA targeted to the TAR sequence has also been shown to inhibit HIV-1 replication in cell lines (99).

RNA decoys containing the HIV-1 TAR structure have been studied extensively as inhibitors of HIV-1 transcription (56, 100-102). TAR decoys specifically inhibit Tat-activated HIV-1 transcription in a cell-free transcription system (100, 101), and appear to function by interacting with Tat-containing RNA polymerase II transcription complexes that assemble on the HIV-1 promoter (56). Overexpression of TAR decoys rendered a T cell line resistant to HIV-1 replication (103, 104). In addition to inhibiting HIV-1 replication, TAR decoys also inhibited HIV-1 RNA expression as assessed by northern blot (102). Polymerization of TAR decoys by expressing multiple tandem copies of the TAR sequence on a single RNA molecule may increase the efficiency with which TAR decoys inhibit HIV-1 transcription (105) and viral replication (106, 107). A potential problem regarding expression of RNA decoys within cells is a relatively short half-life due to the action of ribonucleases. The stability of TAR decoys in cellular extracts increases dramatically when TAR decoys are circularized, yet circular TAR decoys retain their ability to inhibit HIV-1 transcription (101). Circular TAR decoys, however, have not yet been tested within cells for their ability to inhibit viral replication.

In comparison to single gene therapy approaches, combination approaches that target the Tat/TAR interaction can be more efficient at inhibiting viral replication. For example, expression of a combination of anti-sense *tat* RNA and polymeric TAR decoys blocked HIV-1 gene expression and viral replication more efficiently than expressing either alone (108). A dual function anti-*tat* gene that produced a polymeric TAR RNA linked to an anti-sense *tat* RNA sequence was shown to efficiently inhibit HIV-1 replication in cell lines as well as in primary human peripheral blood mononuclear cells (109). Expression of this anti-*tat* gene was regulated by the HIV-1 long terminal repeat such that it was only expressed in HIV-1-infected cells (109). This gene was effective at inhibiting replication of laboratory strains of HIV-1 as well as patient isolates (109, 110). It also inhibited HIV-1 replication when it was transduced *ex vivo* into peripheral blood mononuclear cells isolated from patients with AIDS (111). Transduction of this gene into human CD34+ hematopoietic progenitor cells derived from cord blood resulted in the differentiation of CD4+ T cells that were resistant to HIV-1 infection (112). This type of genetic manipulation of stem cells such that they produce T cell progeny that are resistant to HIV-1 infection could be a powerful gene therapy approach to combating HIV-1 infection.

4.2. Rev and RRE

The binding of Rev to the RRE and the subsequent Rev-dependent transport of RRE-containing mRNA to the cytoplasm is essential for viral replication. Therefore numerous approaches to inhibit this binding have been studied. *Trans*-dominant Rev mutants (113-121), chimeric Rev molecules which inhibit nuclear export of RNA (122), antisense RRE sequences (123-125), RNA decoys (126-131), ribozymes which selectively cut *rev* (98, 132) and intracellular antibodies which bind to Rev (133-136) have all been evaluated.

Malim and colleagues developed RevM10, the first *trans*-dominant Rev mutant in 1989 (113). Subsequently a number of studies have demonstrated that expression of *trans*-dominant Rev mutants inhibited HIV-1 replication in T cell lines and primary T cells challenged with primary HIV isolates (114-118, 121). A recent report described transduction of RevM10 into CD34-enriched hematopoietic progenitor cells from umbilical cord blood or peripheral blood. These transduced cells gave rise to mature T-cells in SCID-hu mice that were resistant to HIV-1 replication (137). A phase I clinical trial in AIDS patients is underway to study the safety of administering lymphocytes expressing RevM10 to patients (119, 120).

Recently, a chimeric protein consisting of the wild-type Rev protein covalently linked to a mutated NS1 protein of the Influenza A virus (NS1RM-Rev), was found to inhibit nuclear export of HIV-1 mRNA (122). Because oligomerization of Rev seems to be important for Rev function the chimeric NS1RM-Rev protein may display improved oligomerization compared to *trans-dominant* Rev mutants such as RevM10. In comparative studies the NS1RM-Rev protein has been shown to be at least as effective as the *trans-dominant* mutant RevM10 (122).

Inhibition of HIV-1 by antisense oligodeoxynucleotide sequences to the RRE has been described as an effective method for antiviral therapy (123, 124). However, the virus typically breaks through after growth in culture. Nevertheless, at low multiplicity of infection, stable expression of antisense RRE has been shown to confer resistance to breakthrough growth of virus for up to 5 weeks (125).

Another approach to achieve Rev suppression is to overexpress the RRE target sequence. Lee and colleagues demonstrated that overexpression of a 45-nucleotide chimeric tRNA-RRE transcript, suppressed HIV-1 in more than 90% of expressing T cells (127). However, due to concerns that viral RNA sequences may bind and interfere with the function of essential cellular factors a "minimal" 13 nucleotide RNA decoy, corresponding to the authentic Rev binding domain has been constructed and demonstrated to confer resistance to HIV-1 replication in T cell lines (128) as well as primary T-cells (131). Transient protection has also been shown in T-cells transduced with adeno-associated virus (AAV) vectors expressing RRE decoys (129) . Recently, CD34+ cells have been transduced with the minimal 13 nucleotide RRE decoy. The macrophage progeny of these progenitor cells were challenged with HIV and shown to be resistant to replication of macrophage-tropic strains of HIV-1 (126).

Intracellular immunization against Rev has also been studied. Expression of an anti-Rev single-chain antibody (SFv) inhibited HIV-1 replication in human cells (133-135). This intrabody localized to the cytoplasm where it appeared to exert its effects by sequestering wild-type Rev (134) and accelerate its degradation (136). Delivery of SFv to primary T-cells and alveolar macrophages by an AAV vector was shown to effectively inhibit infection with clinical and laboratory isolates of HIV-1, including a multidrug-resistant isolate. A combination approach using SFv and RRE decoys lead to synergistic inhibition (138) and a clinical trial has been proposed based on these findings.

A ribozyme which targets the *rev/env* coding region of HIV-1 has been tested in T cells. This construct protected the T cells from selected strains of HIV-1 (139). Furthermore, ribozymes targeting the *rev* gene of HIV has been successfully used for temporary HIV inhibition in T cell lines (98, 132).

4.3. Nef

The Nef protein, an early gene product which is important for maintenance of high viral loads and the development of AIDS (74), functions to downregulate CD4+ and MHC-I expression on infected cells through endocytosis, thus evading the protective effect of CTLs (75, 78, 79) . Clinical observations on patients infected with HIV-1 strains that contain deletions in their *nef* gene show that these individuals have remained asymtomatic with very low viral loads for 10-14 years (140). This deletion therefore seems to render the virus far less pathogenic, suggesting that *nef* might be a good target for gene therapy. Larsson and co-workers used antisense oligonucleotides and ribozymes to target a conserved 14-nucleotide region in the *nef* gene. They demonstrated a dose-dependent repression in HIV-1 replication in peripheral mononuclear blood cells after administration of the antisense oligonucleotide to the cell culture medium (141). A hammerhead ribozyme targeted at the same site was also shown to effectively repress HIV growth in T-cell lines (141). The conserved *nef* site thus seems to be an attractive new target for HIV-1 gene therapy.

4.4. The *pol* gene

Ribozymes with different *pol* targets have been described. Yu and colleagues constructed a hairpin ribozyme targeted to cleave a conserved region in the *pol* gene. T cells transduced with a retroviral construct encoding this ribozyme gene exhibited significant inhibition of different strains of HIV. Greater protection was shown when the ribozyme expression was driven by a pol III promoter as compared to a pol II promoter (142).

4.4.1. Reverse transcriptase

The RT enzyme is effectively inhibited by pharmacologic agents such as nucleoside and non-nucleoside analogues. In addition intracellular immunization against HIV-1 has been accomplished by intracellular expression of antibody fragments directed against RT (143). Cells expressing the antibody fragments were resistant to infection with laboratory and clinical isolates of HIV-1 and HIV-2. The potential advantage of this approach is that it inhibits viral replication prior to integration into the host genome, thereby allowing possible resolution of the infected state (143).

Another method is being developed for inhibiting RT by using antisense oligonucleotides. Results indicate that antisense oligonucleotides to the U5 region of the viral RNA inhibits RT activity *in vitro* (144). Studies on the efficacy of this approach in cells have not yet been published.

4.4.2. Integrase

In order for the viral cDNA to be transcribed it must be integrated into the genome of the host cell, a process that requires viral integrase, an enzyme encoded within the *pol* gene. This integration is dissimilar to any reaction known to be involved in the normal functioning of the host cell, making this enzyme an attractive target for viral inhibition (145). Inhibition of integrase expression has been accomplished in *Escherichia coli* by using a specific ribozyme molecule which recognizes a GUC sequence in the integrase RNA (146). Intracellular expression of single-chain variable antibody fragments which bind to integrase prevented productive HIV-1 infection in both T-cell lines as well as peripheral blood mononuclear cells (147) presumably by specifically neutralizing the enzyme prior to integration (147).

4.4.3. Protease

The HIV protease, a product of the *pol* gene, is essential for the life cycle of the virus by cleaving the Gag-Pol polyprotein into structural proteins and viral enzymes. Recent success in pharmacologic inhibition of HIV-1 protease has sparked interest in this enzyme and its potential inhibition by using gene therapy. *Trans*-dominant mutant protease has been constructed and shown to prevent protease activation and virion maturation (148). Junker and co-workers showed that T-cell lines which constitutively expressed the mutant protein had dramatically reduced HIV replication when compared to cells expressing the wild-type protein and this approach was also shown to be effective against protease-resistant isolates (149). Another approach to inhibit the protease through competitive inhibition has been taken by Serio and colleagues who overexpressed a chimeric Vpr molecule which contained a protease cleavage site (150) (see 4.7, Vpr) .

4.5. The *gag* gene

The products of the *gag* gene exist in a highly multimerized form in the mature HIV virions. Therefore, they may represent attractive targets for inhibition by *trans*-dominant mutants (151). A number of mutant Gag proteins have been generated that can interfere with the production of infectious viral particles. High-level cellular expression of such Gag mutants confers partial resistance to HIV-1 replication when cells are challenged with a wild-type virus (151). Other methods of *gag*-suppression have been studied, such as antisense sequences complementary HIV-1 sequences in the 5' leader-*gag* region (152, 153). In T cell lines expressing the antisense sequence, HIV-1 replication was inhibitied for 10-20 days. Despite stable expression of the protective sequence, the antiviral effect was not lasting (153). However, by expressing a longer anti-*gag* sequence, a more effective and durable suppression in HIV replication was demonstrated in primary CD4+ lymphocytes (154).

Ribozymes targeted to HIV-1 *gag* transcripts have been developed and transfected into cell lines (155). Subsequent challenge with HIV-1 revealed cleavage of *gag* RNA and substantial reduction of viral production. Importantly, the ribozyme expression had no effect on cell replication and viablity, suggesting that it was not toxic to cells.

A novel antiviral strategy has been designed in which the retroviral *gag* genes are fused to nuclease genes, creating fusion proteins that are destructive to viral components within the assembling virion. This strategy has been referred to as capsid-targeted viral inactivation (156, 157). Schumann and co-workers studied RNase HI from *Escherichia coli* fused to Moloney murine leukemia virus (MMLV) coat protein. They demonstrated that the fusion protein is encapsidated into virions where it is cleaved by the MMLV protease, releasing a functional RNA-cleaving enzyme. This approach reduced virus output by 97-99% in MMLV infected cells (157), but has yet not been studied in HIV-1 infection.

4.6. The *env* gene

The possibility of generating novel gene delivery vectors with tropism for HIV-infected cells may open novel strategies for treatment of persistent and latent infections. Two recent reports of selective targeting of HIV-infected cells expressing viral envelope proteins warrant description (158, 159). Mebatsion et al. generated a rabies virus with altered receptor specificity. By pseudotyping the virus with CD4 and CXCR4-derived proteins they were able to demonstrate that it selectively infected cells expressing HIV-1 envelope proteins (gp120 and gp41). Antibodies to CD4 or the envelope protein

blocked entry of this modified rabies virus (158). Similarly, Schnell and co-workers constructed a recombinant vesicular stomatitis virus expressing CD4 and CXCR4. This virus was unable to infect normal cells but infected propagated on and killed HIV-infected cells that expressed the HIV-1 membrane fusion protein (gp120/gp41) on their cell surface. Moreover, this killing of HIV-1 infected cells controlled the infection in a T-cell line and reduced titers of infectious HIV-1 in the culture by 300-fold to 10,000-fold (159). Conversely, recent reports describe selective targeting of CD4-expressing cells by pseudotyping murine leukemia virus such that it expresses the HIV-1 envelope proteins (160).

Intracellular immunization to HIV-1 has been studied by using a intracellular single chain antibody (intrabody) to gp120 (sFv105) (161). This intrabody reacts with the nascent folded envelope protein (gp160) within the endoplasmic reticulum and prevents cleavage to gp120 thereby inhibiting subsequent transit of the envelope protein to the cell surface. The infectivity of the cells expressing this intrabody was substantially reduced (161), but viral replication was only delayed temporarily (5 days).

Multitarget ribozymes which target several different sites in the gp120 sequence have recently been developed. Some of these constructs target up to nine different conserved regions in the RNA (nonaribozymes) (162). Results from cotransfection experiments in cell lines indicate that antiviral activity can be substantially increased by using several hammerheads with catalytic activity on the same molecule (see 5, combinations of antiviral strategies).

4.7. Vpr

The Vpr accessory protein can be seen as an attractive target for gene therapy because the nuclear localization signal of Vpr mediates transfer of the preintegration complex to the cell nucleus (19), enabling HIV to infect nondividing cells (20-22). The Vpr protein also arrests the infected cells in G2 of the cell cycle, thereby increasing viral production of each infected cell (22). The Vpr protein is packaged into the HIV-1 virions through specific interactions with the Gag polyprotein precursor. The specificity of Vpr for the viral particle can be exploited to deliver antiviral elements and thereby interfere with virus maturation. It has been shown that foreign proteins can be targeted to HIV-1 particles via fusion with Vpr. Wu and colleagues fused a staphylococcal nuclease gene to Vpr, generating a fusion protein which, when coexpressed with its cognate Gag (p55) protein was incorporated into the viral particles where it was enzymatically active (163). Another recent report describes the use of chimeric Vpr proteins containing HIV-specific protease cleavage sites added to the C terminus of Vpr. Interestingly, the chimeric Vpr containing the cleavage sites completely abolished virus infectivity (150). The authors suggested that the mechanism of inhibition was by overwhelming protease activity by a competitive mechanism (150).

4.8. The 5' leader sequence

Hairpin ribozymes which target the HIV-1 5' leader sequence (U5) (164, 165) have been shown to inhibit HIV-1 in T cells lines (166-168). Primary lymphocytes expressing this ribozyme have been shown to be protected from HIV replication (169). Theoretically, all HIV-1 RNAs should be susceptible to the action of this ribozyme since it cleaves the cap structure away from the RNA and capless RNAs cannot be translated into proteins. This ribozyme was recently transduced with a retroviral vector into CD34+ cells from fetal cord blood. Subsequently, macrophage-like progeny derived from these CD34+ cells were shown to resist infection by a macrophage-tropic isolate of HIV-1 (170).

4.9. Packaging sequence ψ

For retroviruses in general, including HIV-1, the efficient packaging of genomic RNA into viral particles requires a specific sequence, termed the ψ (psi) packaging sequence (84, 85). In HIV-1, this region has been shown to be highly conserved, suggesting its feasibility as a target for gene therapy. Deletion of 19 base pairs encompassing ψ between the 5' LTR and the *gag* gene initiation codon results in a virus that is markedly attenuated for replication in human T-cells (85). Sun and colleagues showed that ψ antisense constructs suppressed viral production. They further constructed a ribozyme which cleaved ψ, rendering T-cell clones less susceptible to viral replication when challenged with HIV-1 (171).

4.10. HIV-regulated suicide genes

Another novel approach to achieve selective killing of HIV-infected cells involves the introduction of suicide genes to the genome. The rationale for the use of suicide genes is to eliminate infected cells selectively and thus inhibit the propagation of virus. Thus, to be useful for gene therapy the expression of the suicide genes needs to be limited to infected cells. The herpes simplex shutoff gene (*vhs*) encodes a protein which nonspecifically accelerates the degradation of mRNA molecules to facilitate shutoff of host protein synthesis during lytic infections. Hamouda et al. recently tested the ability of *vhs* expressed from the viral LTR promoter to inhibit HIV-1 replication (172). They demonstrated that the protein inhibited HIV-1 replication more than 44,000-fold when compared to a nonfunctional mutant protein.

Another potential suicide gene is the herpes simplex type 1 thymidine kinase gene (HSV-TK) under the transcriptional control of HIV-1 LTR (173) or modified HIV-2 promoters (174). Acyclovir was added to the medium and upon infection with HIV-1 the HSV-TK gene was transcribed and thus phosphorylated acyclovir to its toxic metabolite which lead to cell death. This approach has been shown to halt the spread of HIV-1 in cell cultures upon addition of acyclovir (173) or ganciclovir (174) in concentrations routinely achieved in the clinical setting. Similar studies have been conducted by expressing this gene from replication-defective adenoviral vectors (175). Other genes have also been studied for selective killing of HIV-infected cells, such as the Diphteria Toxin A under the transcriptional regulation of the Tat and Rev proteins (176, 177).

A hybrid molecule of the human CD4 and a modified version of the *Pseudomonas* exotoxin A (CD4-PE40) has been designed to kill HIV-infected cells (178). The molecule is designed to bind to infected cells by CD4-gp120 interaction at the cell surface. After uptake by the cell, the exotoxin inhibits protein synthesis, leading to cell death. CD4-PE40 has been studied in cell lines infected with laboratory strains of HIV-1 and has been shown to be efficatious. The CD4-PE40 is less active in the presence of primary isolates, however, since continuous presence of the molecule is required to inhibit HIV-1 replication (178).

5. COMBINATIONS OF ANTIVIRAL STRATEGIES

Although most studies have focused only on single antiviral genes, combinations of antiviral elements

in a single vector often confers added benefit as discussed below. Given the success of antiviral drugs, future clinical trials gene therapeutic strategies are likely to be used in combination with already established antiviral drug regimens. Relatively few reports, however have focused on the efficacy of such combinations. The addition of pharmacologic inhibitors of NF-κB has been recently studied in cells stably expressing an anti-Tat sFv antibody (179). Theoretically this combination should result in cooperative suppression in LTR-driven gene expression and replication. On challenge with HIV-1 it was shown in both T cell lines and primary T cells that the combined treatment resulted in more profound and durable antiviral action than was seen with either treatment alone (179).

5.1. Ribozymes and RNA decoys

A combination of ribozymes and RNA decoys has recently been described. A ribozyme with an RRE or TAR sequence coupled to the stem loop of each ribozyme was shown to exhibit dual function *in vitro* (180). A combination of TAR decoys and *gag*-specific ribozymes has been shown to suppress HIV-1 and simian immunodeficiency virus in T cell lines (181). Another chimeric molecule consisting of an RRE RNA decoy fused with a ribozyme directed at the HIV-1 U5 sequence has been constructed. Subsequent challenge of T cells transduced with this construct revealed much greater protection in cells expressing the chimeric construct as compared to the ribozyme molecule alone (182). A triple-copy vector encoding the U5 ribozyme, a ribozyme against the *env/rev* region, and a RRE decoy was recently shown to confer an even higher level of resistance to T cells against diverse clades of HIV-1 (183).

5.2. *Trans*-dominant mutant combinations

Increased suppression of HIV-1 replication may be achieved by combinations of two *trans*-dominant mutant proteins. A novel fusion protein, Trev, which joins Tat and Rev *trans-dominant* mutant sequences has been constructed and shown to inhibit both Tat and Rev activities in T-cell lines and primary T-cells challenged with HIV-1 (184, 185). When expressed simultaneously, Tat and Rev *trans-dominant* mutants resulted in additive or even synergistic inhibition of HIV-1 when compared to the Rev mutant alone (186). This paradigm also seems to apply to other combinations; for example Gag dominant mutants and *gag* antisense sequences are more effective with combined expression of TAR-decoys (107).

5.3. Multitarget ribozymes

A novel approach to HIV-1 inhibition has been taken by Chen and colleagues who constructed several multitarget ribozymes with variable number of hammerhead motifs (mono-, di-, tetra-, penta-, and nonaribozymes), each of which was targeted to cleave HIV-1 *env* RNA at up to nine conserved sites (162). In cotransfection experiments in cell lines, activity of the ribozyme construct roughly paralleled the number of catalytic motifs on the molecules. More recent work in stably transduced T-cell lines has confirmed that the multimeric ribozymes confer an added resistance to HIV replication (187). Another modification of ribozymes using so-called "shotgun" ribozymes against HIV has been described (188). These are multiple ribozymes, flanked by *cis*-acting ribozymes at both their 3' and 5' ends. Upon transcription multiple ribozyme molecules are trimmed at their 3' and 5' ends, resulting in release of multiple independent ribozymes, each of which can be targeted differently. RNA decoys (RRE and TAR) can be added to the *cis*-acting ribozymes, further enhancing the antiviral activity of this technique by sequestering Tat and Rev *in vitro* (180). Despite promising results *in vitro*, however, these ribozymes have still not been studied *in vivo* (188).

5.4. Other combinations

Dual inhibition by antisense oligonucleotides to both the TAR and the polyadenylation signal has been shown to reduce the production of infectious HIV-1 by more than 99% in cell lines (99). Similarly, expression of multitargeting *tat/rev* antisense sequences has been shown to result in stable resistance to HIV in monocytic cells (189).

Recent data from primary T-cells and alveolar macrophages shows that combination of anti-Rev single chain immunoglobulin (SFv) and RRE RNA decoys delivered by AAV vectors results in synergistic inhibition of both laboratory and clinical isolates of HIV-1 (138). Although proviral sequences could be detected following challenge with HIV-1, viral production (p24) was 1000-fold lower in the cells harboring the combination.

The combination of an anti-Rev SFv which targets the Rev activation domain and a ribozyme which specifically targets RRE has recently been developed (190). A modest additive effect of the combination was demonstrated in T-cell lines.

6. PERSPECTIVE

Research on the pathogenesis of HIV-1 infection and the HIV-1 life cycle has lead to the identification of numerous potential viral targets for gene therapy. As summarized here, gene therapy approaches directed at several of these viral targets have been successful in inhibiting HIV-1 replication in cultured human cells, although clinical trials to study the safety and efficacy of gene therapy for HIV-1 infection are only beginning. Although this review has focused on viral targets, gene therapy directed at cellular targets also has potential. For example, people who have homozygous deletions in the HIV-1 coreceptor CCR-5 gene display resistance to HIV-1 infection (191). These people have normal immune systems and no obvious abnormal phenotype, suggesting that the CCR-5 gene is non-essential. Therefore gene therapy approaches designed to knock out expression of CCR-5 expression in CD4+ cells from HIV-1 infected individuals could potentially be useful in treating HIV-1 infection. Gene therapy approaches designed to enhance the immune response to HIV-1 infection also have potential (192, 193).

Because of recent advances in our understanding of the pathogenesis and treatment of HIV-1 infection, this is an exciting time to care for patients infected with HIV-1. Although there is no cure for AIDS, we now have drugs that are effective against HIV-1, and we can offer patients hope. Combinations of new drugs have been shown to reduce the occurrence of clinical events (development of opportunistic infections and death) in patients infected with HIV-1 (194). Despite these advances, there is clearly a need to develop better therapies. Although triple drug regimens consisting of reverse transcriptase inhibitors and protease inhibitors suppress plasma virus levels to low or undetectable levels in a large percentage of patients, not all patients respond to the same extent (3), and in those patients that respond, the duration of the response is not known. Vigorous research efforts should continue on the development of more effective drugs against HIV-1 as well as on the development of gene therapy approaches. In addition to potentially providing new therapies for HIV-1 infection, continued research on gene therapy for HIV-1 will likely provide new insights into the pathogenesis of

HIV-1 infection. Advances in our understanding of gene therapy for HIV-1 infection may be helpful in developing gene therapy approaches to other human diseases.

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