3
DNA Vector-Mediated siRNAs or Short Hairpin RNAs

siRNA-directed silencing by transfection is limited in mammals by its transient nature. To overcome some of the shortcomings of transfecting chemically synthesized siRNAs into cells, several groups have developed DNA vector-mediated mechanisms to express substrates that can be converted into siRNAs in vivo (Kennerdell and Carthew 2000; Tavernarakis et al. 2000; Svoboda et al. 2001; Brummelkamp et al. 2002a; Lee et al. 2002; McManus et al. 2002; Miyagishi and Taira 2002; Paddison et al. 2002a,b; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002; Kawasaki and Taira 2003) (Fig. 3). Alternatively, small RNA molecules might also be expressed in cells following the cloning of siRNA templates into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small-nuclear RNA (snRNA) U6 or the human RNase P RNA H1 (Medina and Joshi 1999; Paule and White 2000; Myslinski et al. 2001).

Two approaches have been used to express siRNA species through constructs that are driven by RNA pol III. In the first approach, the sense and antisense strands of the siRNA duplex are expressed from different, usually tandem, promoters (Fig. 3) (Lee et al. 2002; Miyagishi and Taira, 2002; Yu et al. 2002). In vivo, these strands come together to form a 19-nt duplex with 4-nt overhangs from the pol III-termination signal. The second approach uses Dicer to express and process short hairpin (sh)RNAs into siRNAs (Fig. 1). Dicer is required for the processing of pre-*let7* RNA—which is a structured, approximately 70-nt hairpin—into the mature, 22-nt active miRNA species (Reinhart et al. 2000; Grishok et al. 2001; Hutvagner et al. 2001; Knight and Bass 2001; Hutvagner and Zamore 2002). H1 RNA–pol III-based shRNA expression vector has been used to produce hairpin RNA with a 19-nt stem and a short loop (Brummelkamp et al. 2002a; Fig. 3). This system was used to inhibit the expression of E-cadherin (CDH1) and p53 with a comparable efficiency to siRNA transfection. Using RNA based on the *let7* precursor, *luciferase* mRNA has been targeted for degradation by including a 32-nt *luciferase*-complementary sequence in the stem of the hairpin (Paddison et al. 2002b). When transfected into *Drosophila* S2 cells, they found that although *let7*-basedpre-*let7* RNA structures could target the *luciferase* mRNA, the most effective inhibitors had a simple hairpin structure with full complementarity in the stem. To express hairpin RNA in mammalian cells, they developed a U6 RNA–pol III-based expression system, which used a 29-nt sequence complementary to the luciferase gene and an 8-nt loop.

Although most expression systems use either the U6 or H1 promoter, an expression system that uses the transfer (t)RNA^{Val} promoter was described. shRNAs that have been generated using this expression system show a strong cytoplasmic localization and are efficiently processed by Dicer into siRNAs (Kawasaki and Taira 2003).

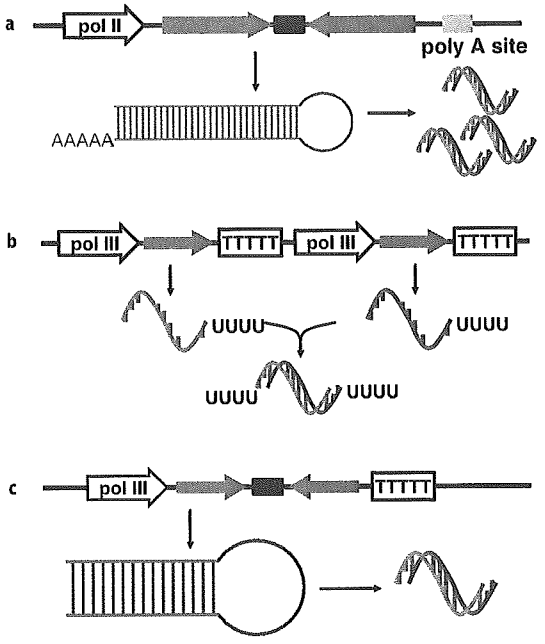

Fig. 3a–c Endogenous expression of short-interfering RNAs (siRNAs). **a** Long hairpin RNA expressed from an RNA polymerase (pol II) promoter yields a population of siRNAs with various sequence specificities. **b** An expression cassette for sense and antisense siRNAs using the tandem pol III small-nuclear RNA (snRNA) promoter. The preferred target site, which has been selected for optimal vector design, is indicated at the *bottom*. **c** A single pol III cassette for expressing hairpin RNAs that are subsequently processed to siRNAs. In this case, transcript synthesis is initiated with a +1 guanosine, and the 3′ end of the sense strand is joined by short oligonucleotide loops with the antisense strand

Some investigators have employed viral vectors in order to facilitate the introduction of siRNA-expressing cassettes into cells. Human immunodeficiency virus type-1 (HIV-1)-based lentivirus vectors have attracted particular attention in this regard. These vectors exploit the ability of HIV-1 to infect non-dividing cells (Weinberg et al. 1991; Bukrinsky et al. 1992; Lewis et al. 2002). HIV-1-based lentivirus vectors retain this central characteristic and, as such, are particularly suitable for the transduction of non-dividing cells, such as neurons and hematopoietic progenitor cells (Naldini et al. 1996).

Lentivirus vectors expressing shRNAs have been shown to promote specific gene silencing in primary dendritic cells (Stewart et al. 2003), while CD8-specific shRNAs expressed from an HIV-1-based vector were capable of silencing CD8 expression both in vitro and in vivo (Rubinson et al. 2003). Collectively, these studies illustrate the broad utility of RNAi for the silencing of viral and cellular processes in vitro and in vivo.

4
Inhibition of HIV-1 Replication by RNAi

The introduction of combination antiretroviral therapy has resulted in a remarkable improvement of the life expectancy of individuals infected with HIV and has significantly reduced their likelihood of developing AIDS. However, despite this progress, HIV infection remains incurable. Toxicity problems associated with current drug therapies and the emergence of drug resistance clearly indicate the need for alternative therapeutic approaches. Retroviral infection with HIV results in the stable integration of proviral DNA into the genome of target cells, and can therefore be viewed as an acquired genetic disease. Thus, the modulation of HIV replication by the expression of antiviral genes might be a therapeutic option for HIV infection. Baltimore (1988) was the first to suggest the concept of gene therapy as an intracellular immunization against HIV. Recently, numerous anti-HIV gene-therapy approaches have been developed and tested in clinical trials. These strategies can be divided into two main categories: first, the genetic modification of HIV target cells or their progeny in order to inhibit HIV replication and second, the genetic modification of cells in order to generate an immune response against HIV or HIV-infected cells. The latter category can be viewed as gene therapy-based immunotherapy and will not be discussed further in this review.

The inhibition of HIV replication involves the transfer of genetic material into HIV-1 target cells or their progenitors ($CD4^+$ T cells or hematopoietic stem cells). A typical gene-therapy approach for HIV-1 infection is schematically depicted in Fig. 4. HIV-1 is well suited for target RNAi because dsRNAs act at multiple steps during the HIV-1 replication cycle (Fig. 4). The inhibitory proteins that are used against HIV act intracellularly and include antibody fragments, single-chain variable fragments, transdominant negative HIV, and cellular proteins. Most of these approaches target viral RNA or proteins. Additional cellular factors that are prerequisites for HIV infection or replication are also potential targets for anti-HIV gene therapy. A number of studies have reported that the transient transfection of siRNAs directed to several HIV-1 genes (*HIV-1 LTR, gag, vif, nef, tat* and *rev*) induced pre-integrated HIV-1 RNA degradation and consequently reduced HIV-1 antigen production by infected cells (Brummelkamp et al. 2002a; Capodici et al. 2002; Coburn and Cullen 2002; Jacque et al. 2002; Lee et al. 2002; Lewis et al. 2002; Novina et al. 2002; Paul et al. 2002; Surabhi and Gaynor 2002; Yamamoto et al. 2002; Yu et al. 2002; Song et al. 2003a). Lee and colleagues, and Banerjea and co-workers, demonstrated that a psiRNA approach can be used to inhibit the expression of HIV-1 *rev* and/or *tat* transcripts in transient transfections (Lee et al. 2002) or from lentiviral-transduced hematopoietic progenitor cells (Banerjea et al. 2003). In this approach, the vectors contain two tandem human U6 snRNA promoters followed by 21-mers encoding sense and antisense siRNAs. In co-transfection experiments, psiRNAs that were co-transfected with the HIV-1 pNL4-3 provi-

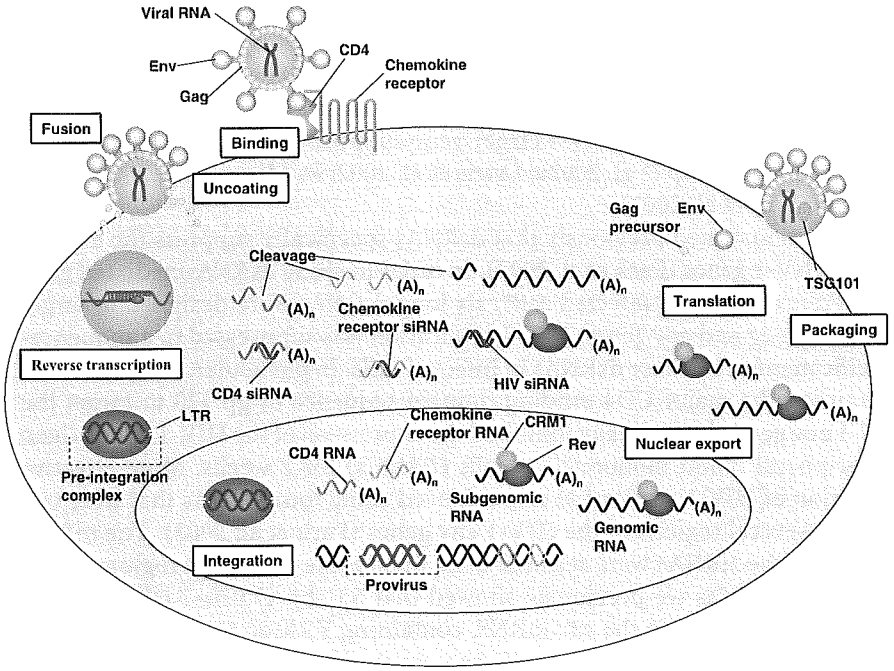

Fig. 4 RNA interference (RNAi) target sites in the human immunodeficiency virus type-1 (HIV-1) replication cycle. Short-interfering RNAs (siRNAs) that target HIV-1 RNA might induce the cleavage of pre-integrated RNA or interfere with post-integration HIV-1 RNA transcripts and block progeny virus production. siRNAs targeting CD4, CXCR4, or CCR5 RNA transcripts inhibit virus attachment to the CD4 receptor or chemokine receptor-mediated HIV-1 fusion and entry. As cleavage of the messenger RNA (mRNA) target requires a high degree of complementarity between the siRNA and its target sequence, heterogeneity in the virus population might prevent efficient silencing of some virus variants by specific siRNAs

ral DNA inhibited HIV-1 p24 antigen expression by up to 4 logs (Lee et al. 2002). This strong inhibition was achieved by simultaneously targeting two essential sites (*rev* and *tat*). Synthetic siRNAs targeted to HIV-1 *rev* and *tat* mRNAs were also shown to inhibit HIV-1 gene expression and replication in both human T cell lines and primary lymphocytes (Coburn and Cullen 2002).

Additional studies have demonstrated that siRNAs act at a later stage of the HIV-1 life cycle, causing post-integration degradation of HIV-1 RNA transcripts (Jacque et al. 2002; Lee et al. 2002; Novina et al. 2002).

The HIV-1 gag gene is expressed during the later steps of HIV-1 replication and encodes the gag-precursor protein, which is proteolytically cleaved into p24 and other polypeptides. p24 forms the HIV-1 core and functions by uncoating and packaging viral RNA. Novina et al. (2002) transfected cells with anti-gag siRNAs, exposed them to HIV-1, and observed a decrease in the in vitro production of p24. Co-transfections by Jacque et al. (2002) of a proviral

HIV-1 clone, 19-bp stem siRNAs directed against other HIV-1 genes (such as *vif* and LTR-*TAR*), and insertational mutagenesis of *nef* by a green fluorescent protein (GFP) gene, showed a significant suppression of virus production compared with non-transfected cells. Such siRNA- or shRNA-expression systems, if stable, might allow long-term target-gene suppression in cells (Naldini et al. 1996; Brummelkamp et al. 2002a; Lewis et al. 2002; Paddison et al. 2002b; Paul et al. 2002; Yu et al. 2002).

We demonstrated previously that dsRNAs specifically suppress the expression of HIV-1 genes (Park et al. 2002). In order to study dsRNA-mediated gene interference in HIV-1 infected cells, six long dsRNAs were designed to target the HIV-1 *gag* and *env* genes. HIV-1 replication was suppressed in a sequence-specific manner by these dsRNAs in infected cells. In particular, the E2 dsRNA, containing the major CD4 binding-domain sequence of gp 120 to target the HIV-1 env gene, dramatically inhibited the expression of the HIV-1 p24 antigen in peripheral blood mononuclear cells (PBMCs) for 2 weeks. More effective inhibition of HIV-1 replication was achieved using four siRNAs that were targeted to several regions of the HIV-1 env genes (Park et al. 2003). The mRNA targets for the siRNAs were selected from the middle of the *env* regions in the HIV-1 genome, as we previously showed that 531-bp (7.070–7.600) E2 dsRNAs complementary to the *env* mRNA-containing V3 loop and the major CD4 binding-domain sequence of gp 120 were more effective inhibitors than those targeted to the *gag* gene. Furthermore, the envelope protein (Env) of HIV-1 mediates functions that are critical to the viral life cycle, including viral attachment to target cells, and fusion of the viral and cellular membranes. We also showed the inhibition of HIV-1 replication in T cells using E2 shRNA directly from a lentivirus vector (Hayafune et al. 2005). On the other hand, we have shown that vif shRNA specifically suppresses the expression of HIV-1 (Barnor et al. in press). The HIV-1-encoded vif protein is essential for viral replication, virion production, and pathogenicity. HIV-1 vif interacts with the endogenous human APOBEC3G protein (an mRNA editor) in target cells to prevent its virions from encapsidation. Previous studies have established targets within the HIV-1 vif gene that are important for its biological function; however, it is important to determine effective therapeutic targets within *vif* because of its critical role in HIV-1 vif-dependent infectivity and pathogenicity. vif shRNAs increased the inhibition of HIV-1 replication in a long-term culture assay.

Rather than targeting the viral RNA, an alternative way of inhibiting virus replication by RNAi is to silence the expression of cellular genes that are critically involved in viral replication. For HIV-1, these targets include the mRNAs encoding the CD4 receptor and the CCR5 or CXCR4 co-receptors. These receptors are essential for attachment of the HIV-1 particle to the cell and for subsequent viral entry. RNAi against the viral RNA does not protect the cell against viral entry. By silencing these receptors, the HIV-1 particle will be unable to attach to, and enter, the cell, thus yielding a form of HIV-1 resistance.

Novina et al. (2002) showed that specific siRNAs that were directed against either CD4 or gag genes were able to prevent HIV-1 replication in MAGI and H9 cells. siRNA targeting rendered the receptor unavailable for virus attachment, thereby inhibiting HIV-1 entry and virus production. However, CD4 targeting might not be a feasible therapeutic approach because of its importance in immune function. By contrast, CCR5, which is the major HIV-1 co-receptor for viral entry into macrophages, might be a potentially useful cellular target, as a 32-bp homozygous deletion of the gene abolishes its function without deleterious immunological consequences and provides protection from HIV-1 infection (Martinez et al. 2002; Qin et al. 2003; Song et al. 2003a).

In this regard, RNAi is a powerful tool with which to determine the role of cellular co-factors in HIV-1 replication. Indeed, the first study to use RNAi in HIV-1 research silenced the expression of TSG-101, which is a component of the class E vacuolar protein-sorting pathway, by means of siRNAs (Garrus et al. 2001). This revealed a critical role for TSG-101 in the budding of HIV-1 virions. Moreover, when a lentivirus-based vector system was used to introduce shRNAs against CCR5 into peripheral blood T lymphocytes, the expression of CCR5 on the cell surface was reduced tenfold, resulting in a three- to sevenfold decrease in the number of infected cells (Qin et al. 2003). Lee et al. (2003) also showed inhibition of HIV-1 replication in macrophages using tat or CCR5 directly from a lentivirus vector. Similarly, siRNAs directed against CXCR4 co-receptors blocked HIV-1 entry, and protected cells from infection and delayed virus replication (Anderson et al. 2003). Another host factor that is important for HIV-1 replication is the transcription factor nuclear factor (NF)-κB to motifs in the long terminal repeat (LTR) promoter of the integrated provirus is required for viral transcription (Surabhi and Gaynor 2002). However, targeting NF-κB is not an appropriate therapeutic option, owing to the important role of NF-κB in cells.

5
siRNA Agents Work as Ligands for Toll-Like Receptors

Surprisingly, recent studies have indicated that siRNAs can induce global up-regulation of the expression of IFN-stimulated genes (Bridge et al. 2003; Jackson et al. 2003; Sledz et al. 2003; Kariko et al. 2004; Persengiev et al. 2004; Fig. 5). This effect was detected with synthetic siRNAs that were transfected into cells, and with siRNAs that were produced within cells by the expression of shRNAs. Both of these papers documented significant non-specific changes in gene expression as a consequence of the delivery of siRNAs. Sledz et al. (2003) observed a 2-fold induction of 52 out of 850 putative IFN-stimulated genes using synthetic siRNAs. By contrast, Bridge et al. (2003) observed a 50-fold induction of the IFN-stimulated gene *OAS1* with one siRNA vector alone, and a 500-fold induction when two vectors were used simultaneously. These results suggest

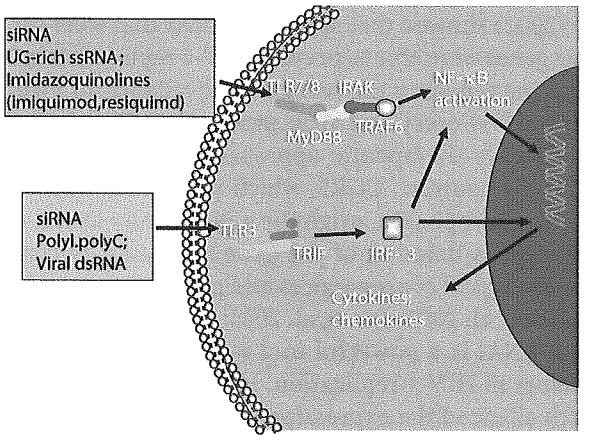

Fig. 5 Toll-like receptor (TLR) signaling pathways. TLRs recognize molecular patterns associated with bacterial pathogens, double-stranded RNA and siRNA for TLR3; siRNA, UG-rich ssRNA, imidazoquinoline and its derivatives for TLR7/8. TLR signaling pathways are separated into two groups: (1) A MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK; and (2) TRIF, which may exist downstream of TLR3/4, and IKKε and TBK1 to mediate the MyD88-independent pathways leading to production of IFN-β and IFN-inducible genes

that the ability to induce the IFN system depends on both the siRNA sequence and the method of delivery. Both groups pointed out that increasing the quantity of the siRNAs enhanced the effect. Furthermore, two recent studies have indicated that the mechanism of the IFN response might include recognition of the siRNAs by Toll-like receptor 3 (TLR3) (Heidel et al. 2004; Kariko 2004). One simple method for limiting the risk of inducing an IFN response is to use the lowest effective dose of shRNA vector, as advocated by Bridge et al. (2003).

Recently, Kim et al. (2004) showed that siRNAs synthesized using the T7 RNA polymerase system can trigger the apotent induction of IFN-α and -β in a variety of cells. The mediators of this response revealed that an initiating 5′-triphosphate was required for IFN induction. These findings have led to the development of an improved method for bacteriophage polymerase-mediated siRNA synthesis that incorporates two 3′ adenosines in order to prevent base-pairing with the initiating Gs, thereby allowing RNase T1 and calf intestine alkaline phosphatase (CIP) to remove the initiating 5′ nucleotides and triphosphates of the transcripts.

6 Virus Escape from RNAi

When profound inhibition of virus replication is obtained by means of RNAi technology, the possibility of viral escape must be considered. This potential

problem is particularly relevant for viruses that exhibit significant genetic variation due to an error-prone replication machinery.

This risk might be more severe for RNA viruses and retroviruses than for DNA viruses. The variability of HIV caused by its error-prone reverse transcriptase has been shown to generate mutations in the gene being targeted, thus allowing it to rapidly evade siRNAs (Boden et al. 2003). Similar results were observed for RNAi of a poliovirus infection (Gitlin et al. 2002). Synthetic siRNAs against poliovirus inhibited virus production 100-fold; however, the virus titer increased to high levels upon prolonged incubation. Sequence analysis of the progeny virus demonstrated a single escape mutation in the center of the siRNA target sequence. These findings indicate that the point mutation occurred in the middle of the signal target sequences. Two recent studies have demonstrated that transfection with siRNAs containing mismatches to the target sequence in the middle of the siRNA molecules reduces the efficiency of gene silencing (Brummelkamp et al. 2002b; Amarzguioui et al. 2003). These findings suggest that in order for RNAi to durably suppress HIV-1 replication, more potent shRNAs will need to be designed that can target highly conserved regions of the viral genome (for example, *gag* and *pol*) that are essential for the viral life cycle. Alternatively, RNAi constructs co-expressing multiple shRNAs could be developed that simultaneously target different regions of the viral genome, thereby reducing the probability of generating shRNA escape mutants.

7
Using RNAi to Treat Other Viruses

Although many previous studies on RNAi-mediated inhibition have focused on HIV-1, there is a growing body of data addressing the inhibition of other animal and human viruses. These include RNA viruses such as hepatitis C virus (HCV), poliovirus, Semliki Forest virus (SFV), influenza virus A, rhesus rotavirus (RRV), and Rous sarcoma virus (RSV), and DNA viruses such as human papillomavirus type 16 (HPV-16) and hepatitis B virus (HBV). In most of these studies, the RNAi machinery was directly targeted towards the viral RNA using synthetic siRNAs.

Hepatitis induced by HBV or HCV is a major health problem. At present, hundreds of millions of individuals are infected worldwide. Although there is an effective vaccine against HBV, it is only useful for the prevention of viral infection. There is no vaccine for HCV. Hepatitis caused by these two viruses has therefore been an important target for potential RNAi therapy.

The first demonstration of RNAi efficacy against a virus in vivo involved the hydrodynamic co-delivery of an HBV replicon and an expression unit encoding an anti-HBV shRNA in mice. HBV is a member of the family Hepadnaviridae and has a 3.2-kb circular dsDNA genome. During infection, four RNAs are transcribed, which encode the coat protein (CP), polymerase (P),

surface antigen (S), and transactivator of transcription (X). HBV production in Huh-7 cells was shown to be reduced by up to 20-fold through the transfection of a vector-expressing shRNA against the X mRNA (Shlomai and Shaul 2003).

Inhibition of HBV in the liver of mice was achieved through the co-transfection of HBV DNA and shRNA-expressing plasmids (McCaffrey et al. 2003), which resulted in a six fold decrease in the amount of secreted HBV surface antigen in the serum. This small-animal model of human infectious disease shows that it is possible to use RNAi as a potent antiviral therapy in mammals.

HCV is a major cause of chronic liver disease, which can lead to liver cirrhosis and hepatocellular carcinoma (Reed and Rice 2000). The HCV genome is a positive-strand RNA molecule with a single open reading frame encoding a polyprotein that is processed post-translationally to produce at least 10 proteins. HCV is a member of the family Flaviviridae and has a (+) single-stranded (ss)RNA genome.

Subgenomic and full-length HCV replicons that replicate and express HCV proteins in stably transfected human hepatoma-derived Huh-7 cells have been used to study the effects of various antiviral drugs (Lohmann, et al. 1999; Pietschmann, et al. 2001; Ikeda et al. 2002). Several groups have now tested the efficacy of the siRNA-mediated inhibition of replicon function using these systems (Kapadia et al. 2003; Randall et al. 2003; Wilson et al. 2003). These replicons support HCV RNA transcription and protein synthesis, but do not produce infectious viruses.

siRNAs targeted against sequences in the viral non-structural proteins NS3 and NS5B have been shown to cause profound (up to 100-fold) inhibition of HCV replicon function in cell cultures (Kapadia et al. 2003; Randall et al. 2003; Seo et al. 2003; Wilson et al. 2003). Furthermore, the internal ribosomal-entry site (IRES) in the well-conserved 5′ UTR of the HCV RNA has also been a good target. Both siRNAs and shRNAs have been reported to inhibit HCV replicon function in cells (Seo et al. 2003; Wilson et al. 2003; Yokota et al. 2003; Hamazaki et al. 2005).

In another in vivo study, siRNAs were used to treat fulminant hepatitis induced by an agonistic Fas-specific antibody in mice (Song et al. 2003b). Fas-mediated apoptosis of hepatocytes can be triggered by HBV and HCV infection. Infusing siRNAs targeting Fas mRNAs into the tails of the mice blocked this self-destructive inflammatory response of the liver. These findings indicate that major hurdles remain before this therapy can be applied to humans.

As with HIV therapeutics, delivery of the siRNAs or shRNA vectors is the main challenge for the successful treatment of HCV.

Generally, human influenzal lesions are local infections that remain in the upper portion of the respiratory tract and do not proceed to pneumonia. Nevertheless, in high-risk patients, cases of influenzal pneumonia have been reported in which expansion of the virally infectious focus is observed in a pulmonary lesion. Furthermore, such cases are often accompanied by a secondary bacterial pneumonia. The influenza virus belongs to the family Orthomyxoviridae and

has a (–) ssRNA genome. Its genome is composed of eight separate segments. The proteins encoded by the eight segmented genes include HA and NA, as well as the M1 and M2 membrane proteins, which are located on the surface of the envelope. Furthermore, a nucleoprotein complex (RNP) is located at the center of the virus and is composed of the gene RNA, three RNA polymerase subunits (PB1, PB2, and PA) and a nucleoprotein (NP). A non-structural protein (NS) is synthesized from the eighth segmented gene. Amantadine and rimantadine are known antiviral agents for the influenza A virus; however, neither drug can cope with mutants and both have strong side effects (Atmar et al. 1990; Wang et al. 1993). Treatment by an inactivated vaccine has also been attempted; however, the vaccine cannot sustain antibody productivity for a long period and, thus, cannot completely prevent the spread of infection (Hirota et al. 1996).

Ge et al. (2003) showed that siRNAs targeting conserved regions (PA and NP) of the influenza genome inhibited virus production in cell culture and in embryonated chicken eggs. Furthermore, RNAi mediated by PA-, NP-, and PB1-specific siRNAs or shRNAs expressed from DNA vectors prevented and treated influenza A virus infection in mice (Ge et al. 2004). In addition, Tompkins et al. (2004) showed that the administration of influenza-specific siRNAs decreased lung virus titers and protected mice from lethal challenge by a variety of influenza A viruses, including the potential pandemic subtypes H5 and H7. This specific inhibition of influenza virus replication requires homology between the siRNAs and gene targets, and is not the result of IFN induction by dsRNAs. For therapeutic applications against the influenza A virus, the siRNAs can be administered via intranasal or pulmonary routes. RNAi is more potent than the antisense approach (Mizuta et al. 1999), and the evaluation of this technology as a treatment for the influenza virus through human clinical trials is expected to take place in the near future.

8
Concluding Remarks

While the results obtained to date should be considered preliminary in terms of their application to humans, they do provide strong justification for further investigations into the use of RNAi for the treatment of viruses in a clinical setting. The major problem with using RNAi as a tool to inhibit viral replication is the fact that it is still difficult to predict the effectiveness of specific siRNAs. It is clear from numerous studies that not all siRNAs are equally effective at generating an RNAi response. It has generally been assumed that siRNAs are under the control of the host interference-response mechanism. Recently, some expressed shRNAs have been shown to activate at least one of the arms of the human IFN-response mechanism. In addition, viral escape from RNA silencing is clearly a problem for developing effective RNAi-based antiviral therapy. Furthermore, some viral RNA sequences might be buried within

secondary structures or highly folded regions. Clearly, difficulties concerning the delivery, specificity, and effectiveness of siRNAs remain. However, once these fundamental questions have been addressed, it seems likely that RNAi therapy against viral infections will progress towards clinical trials.

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NEW METHODS AND TECHNOLOGIES

Long-term transgene expression and inhibition of HIV-1 replication by a Cre/loxP-EBNA-1/oriP HIV-1-dependent ribozyme vector: Applications for HIV-1 gene therapy

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ABSTRACT

The cleavage of target mRNA by ribozymes is being exploited as a means of gene silencing in nucleic-acid-based therapies. We previously established an HIV-1-dependent ribozyme-expression vector system, based on Cre-loxP technology with an LTR-*gag-p17* promoter as a molecular switch for use in acute HIV-1 infection. The simultaneous expression of the Cre protein and loxP homologous recombination induced a high level of HIV-1-replication inhibition, but ribozyme expression was transient. In the current study, we overcame this limitation by inserting *EBNA-1* and *oriP* genes from the Epstein-Barr virus (EBV) into the vector. When this plasmid was introduced into HeLa $CD4^+$ cells, we observed long-term expression of both the *EGFP* reporter gene and the ribozyme. Moreover, HIV-1 replication was inhibited in the long-term in transfected cells. These data suggest that the HIV-1-dependent ribozyme-expression vector containing EBNA-1/oriP sequences would be a useful tool in HIV-1 gene therapy applications.

KEYWORDS: *Cre/loxP recombination, EBNA-1/oriP, gene therapy, HIV-1, ribozyme*

INTRODUCTION

In the application of therapeutics to HIV-1 infection, prevention is of greater importance than treatment. With this aim, many antiviral RNA-expression vector systems have been developed for use in anti-HIV-1 gene therapy (Banerjea et al, 2003; Barnor et al, 2004; Boden et al, 2004; Cordelier P et al, 2004; Habu et al, 2005; Mautino and Morgan, 2002; Takaku, 2004). Recently, small RNA molecules such as, siRNAs and snRNAs, have also been shown to affect gene silencing by RNA interference (Boden et al, 2003; Chang et al, 2002; Li et al, 2003).

Increasing the efficiency of transgene expression is of prime importance for human gene therapy (Davis and McNeilly, 2001). The Epstein-Barr Virus (EBV) is an autonomously replicating episomal vector that has been utilized to overcome the problem of rapid elimination of intracellularly delivered plasmid DNA in nonviral gene transfer. EBV is a gammaherpesvirus that is maintained as an episome of approximately 172 kb in size in a small number of resting B cells and epithelial cells in most of the human population. It latently infects human B cells with a high efficiency, after which its linear double-stranded genome circularizes and is sustained as a stable

episome (Hirai and Shirakata, 2001). The EBV replication system is present at a frequency of about 1-100 copies per cell, and maintains a non-covalent attachment to the host chromosome.

The latent origin of replication *oriP* and the viral transactivator protein EBNA-1 are essential components for EBV latent replication and maintenance of the viral genome (Daikoku et al, 2004; Lee et al, 1999). Both elements have been employed for long-term transgene expression in gene-therapy studies (Otomo et al, 2001; Tsujie et al, 2001).

Previously, we described an HIV-1-dependent ribozyme-expression vector capable of achieving site-specific excision of loxP sequences by using the HIV-1 minimal LTR-Cre-loxP system as a molecular switch in an acute HIV-1 infection (Habu et al, in press). However, we were unable to detect long-term expression of the anti-HIV-1 ribozyme. We hypothesized that the length of HIV-1-dependent transgene expression could be significantly increased in mammalian cells by introducing EBNA-1/oriP sequences to the vector.

In this study, we constructed an HIV-1-dependent long-term transgene (RNA ribozyme) expression vector (LTR-*gag-p17*/Cre-loxP-Rz-U5-*EBNA-1-oriP*-EGFP (EOG)) using the EBV replicon system, which was propagated in *Escherichia coli* and transfected into mammalian cells. We measured transgene-expression levels, including *EBNA-1* and *oriP*, in the presence and absence of HIV-1 infectious molecular clone (pNL4-3, Adachi et al, 1986). The potential anti-HIV-1 activity of the expression vector was evaluated with a view to establishing a highly effective therapeutic agent that could be further developed for HIV gene-therapy applications.

MATERIALS AND METHODS

Construction of plasmids

The retroviral vector pLEGFP-C1 (Clontech, Mountain View, CA) was digested with *Nhe* I and *Xho* I to release the DNA fragment encoding enhanced green fluorescent protein (EGFP). This was inserted into the *Nhe* I/*Xho* I sites of pCEP4 (Invitrogen, Carlsbad, CA), which contains *EBNA-1* and *oriP*, to create pCEP4-EGFP. An *Ssp* I fragment containing *EGFP*, *EBNA-1*, and *oriP* was cloned into the *Stu* I sites of pLTR-*gag-p17*-Cre/loxP-Rz-U5 (Habu et al, in press) and ploxP-Rz-U5, which been previously described with a high cleavage affinity (Habu et al, 2002) to yield pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG (Figure 1A) and ploxP-Rz-U5-EOG (Figure 1C), respectively. The control plasmid vector ploxP-Rz-U5-EOG lacks the LTR-*gag-p17*-Cre gene and so does not trigger expression of the ribozyme. A *Pvu* II fragment containing the EGFP-expression unit was excised from pCMV-EGFP previously constructed (unpublished data) and cloned into the *Stu* I sites of ploxP-Rz-U5 (Habu et al, 2002) or pLTR-*gag-p17*-Cre/loxP-Rz-U5 to generate ploxP-Rz-U5-G (Figure 1D) and pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (Figure 1B), which are the EBNA-1 and oriP negative-control plasmids, respectively.

Cell culture and transfections

HeLa $CD4^+$ cells were grown in RPMI 1640 medium (Sigma, Saint Louis, MO) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEK 293T cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. All cells were maintained at 37 °C in a 5% CO_2 atmosphere. HeLa $CD4^+$ and 293T cell transfections were carried out using FuGENE™6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol.

Luciferase assay

Luciferase activity was measured with the PicaGene kit (Toyo-inki, Tokyo, Japan) according to the manufacturer's protocol. The target gene-expressing plasmid pNL4-3-luc (Akkina et al, 1996), lacking an *env* gene and with a firefly luciferase gene replacing the *nef* gene, was co-transfected into HeLa $CD4^+$ cells with the pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG plasmid, which expresses the ribozyme following Cre/loxP homologous recombination. Transfected HeLa $CD4^+$ cells were lysed in 200 µl PicaGene cell lysis buffer for 15 min and then harvested. Cell debris was removed by centrifugation. Centrifuged lysate (10 µl) was added to 100 µl luminous substrate, and the luminescent signal was immediately quantitated with a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany).

Flow cytometry

Transfected HeLa $CD4^+$ cells were trypsinized, washed twice in PBS, and fixed in PBS containing 1% formaldehyde. Direct fluorescence of EGFP was analyzed by FACS Calibur (Clontech). Data acquisition and analysis were performed with CellQuest software (Clontech). Gates for detection of EGFP were established using mock-transfected cells as background.

Fluorescent microscopy

To evaluate the self-replicating function of the loxP ribozyme as an index for stable transgene expression in cells, transfected HeLa $CD4^+$ cells were trypsinized and seeded at a low cell density. Direct fluorescence microscopy of EGFP was carried out at the mitotic stage of cell division, after each passage on days 1, 3, 7, 13, 19 and 25 and the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

RNA isolation and RT-PCR

Total cellular RNA was isolated from transfected HeLa $CD4^+$ cells with the GenElute Mammalian Total RNA kit (Sigma) using the manufacturer's protocol. RNA samples were treated with DNase I (Promega, Madison, WI) according to the manufacturer's instructions. RT-PCR assays were carried out using previously described primers (Habu et al, 2002) and the RT-PCR high-Plus-kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Assay of HIV-1 replication

HIV-1 production was monitored by determining the HIV-1 p24 antigen concentration. The culture medium from HeLa $CD4^+$ cells co-transfected with pNL4-3 and pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG was harvested on days 1,

3, 5, 7, 9, 11, 13 and 15 post-transfection. p24 Gag protein production was detected by the HIV-1 p24 CLEIA assay (Lumipulse, Fujirebio Inc., Tokyo, Japan), according to the manufacturer's protocol (Sakai et al, 1999).

RESULTS AND DISCUSSION

Design and construction of an HIV-1-dependent Cre-expression vector

Tissue-specific gene transfer and expression are crucial for the development of safe and effective gene-therapy protocols. To this end, the HIV-1 LTR can serve as an efficient and inducible promoter dependent on the HIV-1 trans-activation factor, Tat. In a previous study, we constructed the HIV-1-dependent RNA ribozyme expression Cre/loxP vector, pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, which targets mRNAs encoded by the U5 region (548–578) of the LTR (Figure 1E). This vector showed HIV-1-dependent ribozyme expression in HeLa $CD4^+$ cells (Habu et al, 2002; Habu et al, in press), but expression was not long term. Hence, in the current study, we constructed an HIV-1-dependent expression vector containing Cre/loxP and EBNA-1/oriP sequences, with the aim of increasing the duration of transgene expression. Moreover, we inserted the reporter gene *EGFP* to enable visualization of transgene expression (Figure 1A). The plasmid vectors pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (Figure 1B), ploxP-Rz-U5-EOG (Figure 1C), and ploxP-Rz-U5-G (Figure 1D) served as controls. The advantage of this vector system over previously reported ribozyme vector systems (Chang et al, 2002; Li et al, 2003) is that it is not constitutively expressed to trigger off non-specific inhibition, but specifically expressed only in the event of HIV infection.

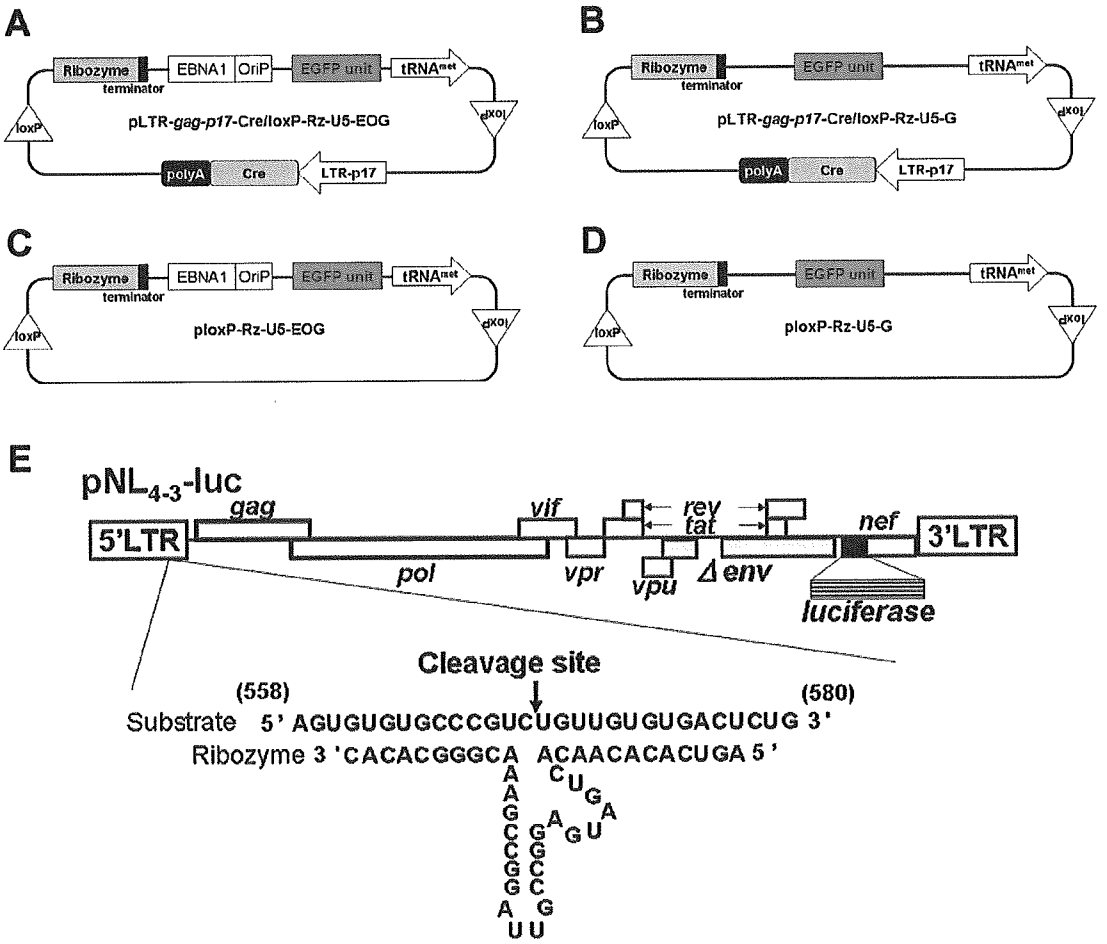

Figure 1. Schematic representation of HIV-1-dependent ribozyme-expression vectors. (**A**) The ribozyme expression (off switch) vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG, containing *EBNA-1* and *oriP* genes for long-term expression. (**B**) Control ribozyme-expression vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, which lacks *EBNA-1* and *oriP* genes. (**C**) Control vector ploxP-Rz-U5-EOG, which lacks the *LTR-gag-p17-Cre* gene. (**D**) Control vector ploxP-Rz-U5-G, which lacks *EBNA-1/oriP* and *LTR-gag-p17-Cre* genes. (**E**) HIV-1 NL4-3 molecular clone pNL_{4-3}-luc containing the luciferase reporter gene, showing the target site and structure of the constructed ribozyme.

Long-term transgene expression of pLTR-gag-p17-Cre/loxP-Rz-U5-EOG in 293T and HeLa CD4$^+$ cells

To characterize the ability of the EBV replication system to effect long-term transgene expression we compared EGFP expression of control vector (pLTR-*gag-p17*-Cre/loxP-Rz-U5-G) with that of the EBNA-1/oriP plasmid pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. EGFP expression in pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected 293T cells was observed for a maximum of 3 days, while EGFP expression persisted for more than 25 days in pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG-transfected 293T cells (Figure 2). As it has been reported that the EBNA-1 protein potentiates gene transcription (Mackey and Sugden, 1999), we measured the enhancement of gene expression by determining EGFP fluorescence intensity. FACS analysis showed that HeLa CD4$^+$ cells transfected with the EBNA-1-containing plasmids demonstrated longer-term EGFP expression (until day 7; Figure 3) than HeLa CD4$^+$ cells transfected with plasmids lacking EBNA-1. This is of therapeutic importance, because EBNA retains the therapeutic molecule to sensor for infective HIV-1 to release the catalytic ribozyme for cleavage.

Measurement of the mean fluorescence intensity (MFI) revealed a twofold increase in the EGFP expression level of HeLa CD4$^+$ cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG compared with pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected cells (data not shown). These results indicate that EBNA-1/oriP sequences mediate efficient and stable replication of transgene expression by enhancing nuclear localization of EBNA-1 (Mackey and Sugden, 1999; Marechal et al, 1999). The nuclear localization of this vector system is of cardinal importance since its function is induced by HIV-1 tat which is nuclear-based.

Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG

The effect of pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG on HIV-1 (pNL_{4-3}-luc) replication was measured in a transient assay following its co-transfection with pNL_{4-3}-luc into HeLa CD4$^+$ cells. At 72 h post-transfection, the luciferase activity of cell lysate was measured as an indirect marker of viral replication. The plasmid vector ploxP-Rz-U5-EOG (Figure 1C), which does not trigger ribozyme expression as it lacks the LTR-*gag-p17*-Cre gene, was used as a control. Our analysis showed a dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG with a maximum inhibitory efficacy of >90% at a vector DNA concentration of 1.67 µg (Figure 4). Control vector alone had no inhibitory effect. This result suggests that the Rz-U5 ribozyme was expressed using Cre/loxP recombination and EBNA/oriP systems in HIV-1 infected cells, and successfully cleaved its target HIV-1 mRNA (Figure 1E).

EBNA-1 mediates long-term ribozyme expression in HIV-1-dependent vector-transfected HeLa CD4$^+$ cells

As long-term ribozyme-U5 (Rz-U5) expression is an important determinant of its efficiency, we compared the level of expression in HeLa CD4$^+$ cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG or pLTR-*gag-p17*-Cre/loxP-Rz-U5-G in the presence or absence of pNL4-3 (Figure 5). RT-PCR analysis showed that Rz-U5 expression persisted for more than 19 days following pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG transfection in the absence of pNL4-3 (Figure 5). By contrast, pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected cells demonstrated Rz-U5 expression for only 3 days. Ribozyme expression was not observed following transfection of either plasmid DNA in the absence of pNL4-3. These results confirm that

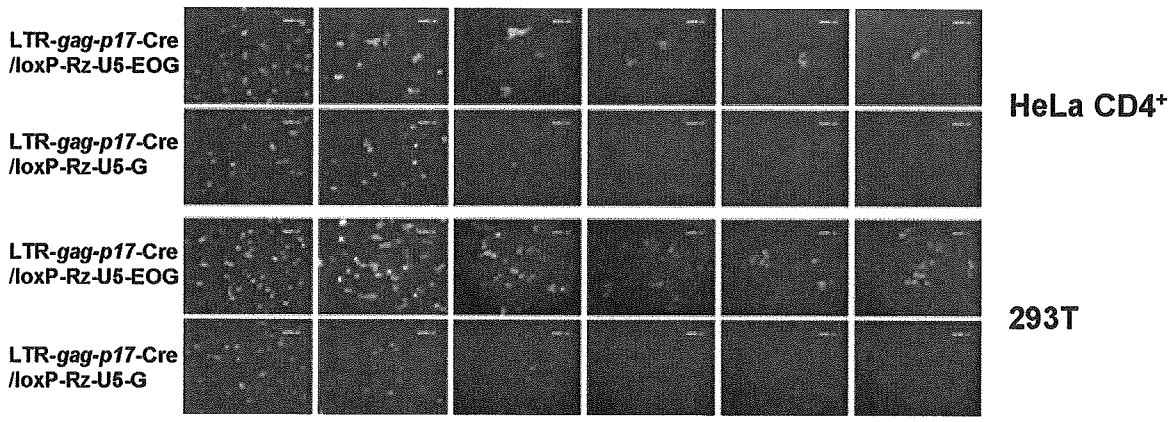

Figure 2. *EBNA-1* and *oriP* increase the duration of EGFP expression in transfected HeLa CD4$^+$ and 293T cells. EGFP expression was evaluated on days 1, 3, 7, 13, 19, and 25 post-transfection.