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Inhibition of HIV-1 multiplication by antisense U7 snRNAs and siRNAs targeting cyclophilin A

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ABSTRACT

Human immunodeficiency virus 1 (HIV-1) multiplication depends on a cellular protein, cyclophilin A (CyPA), that gets integrated into viral particles. Because CyPA is not required for cell viability, we attempted to block its synthesis in order to inhibit HIV-1 replication. For this purpose, we used antisense U7 small nuclear RNAs (snRNAs) that disturb CyPA pre-mRNA splicing and short interfering RNAs (siRNAs) that target CyPA mRNA for degradation. With dual-specificity U7 snRNAs targeting the 3′ and 5′ splice sites of CyPA exons 3 or 4, we obtained an efficient skipping of these exons and a strong reduction of CyPA protein. Furthermore, short interfering RNAs targeting two segments of the CyPA coding region strongly reduced CyPA mRNA and protein levels. Upon lentiviral vector-mediated transduction, prolonged antisense effects were obtained for both types of antisense RNAs in the human T-cell line CEM-SS. These transduced CEM-SS cells showed a delayed, and for the siRNAs also reduced, HIV-1 multiplication. Since the two types of antisense RNAs function by different mechanisms, combining the two approaches may result in a synergistic effect.

INTRODUCTION

Despite years of intensive research and some therapeutic success, AIDS, caused by infection with human immunodeficiency virus 1 (HIV-1) continues to be a major health problem worldwide. New therapeutic or preventive approaches are dearly wanted, and gene therapy carries considerable promises in this respect. Many viral or cellular genes are involved in HIV-1 multiplication and therefore represent potential targets. Indeed, several strategies attempting to interfere with the production or function of such gene products are being tested at pre-clinical or clinical levels [reviewed in (1–3)].

A host protein that has recently attracted attention as a potential target for anti-HIV-1 therapy is cyclophilin A (CyPA). CyPA is a proline *cis-trans* isomerase that was discovered as the cellular ligand of the immunosuppressive drug cyclosporin A [csA; (4)]. Disrupting the CyPA gene in both murine embryonic stem cells and the human Jurkat T-cell line caused no obvious defects, indicating that CyPA is not essential for cell survival or that its function can be compensated for by other factors (5,6). Owing to a specific interaction with the viral capsid (CA) protein, CyPA gets incorporated into HIV-1 virions and is required for efficient viral replication (7–12). This interaction can be disrupted by mutating the N-terminal domain of CA or by treating cells with csA or its non-immunosuppressive analog SDZ-NIM 811. If this occurs in infected cells, the virions produced are of normal morphology and composition and exhibit normal reverse transcriptase activity, but they are devoid of CyPA and show a reduced replication in subsequent target cells. It seems that HIV-1 multiplication is blocked at some step after viral entry but before reverse transcription starts (12), but CyPA may also be required for viral entry (13,14). The functional importance of CyPA in HIV-1 multiplication was demonstrated most directly by inactivating the CyPA gene in Jurkat cells, which resulted in a reduced ability of these cells to produce viruses (6).

However, the feasibility of down-regulating CyPA to retard HIV-1 infection has not yet been explored. Here we describe the use of two different antisense strategies to reduce CyPA biosynthesis. The first approach consists of skipping internal CyPA exons by means of modified derivatives of U7 small nuclear RNA (snRNA). U7 snRNA is the RNA component of the U7 small nuclear ribonucleoprotein (snRNP) involved in histone RNA 3′ end processing [reviewed in (15)]. We have demonstrated that, by inserting appropriate antisense sequences into U7 snRNA, it can be converted from a mediator of histone RNA processing to an effector of alternative splicing (16–18). Here we show that internal exons of the CyPA gene can be skipped efficiently by this approach, resulting in greatly reduced levels of CypA protein. Moreover, HIV-1 multiplication in CEM-SS T-cells that have been stably transduced by lentiviral vectors encoding such antisense U7 snRNAs is significantly impaired.

The other approach used by us to reduce cellular CyPA levels is RNA interference (RNAi), an evolutionarily conserved process found in all higher eukaryotes [reviewed in (19)]. In mammalian cells, RNAi can be induced by 21 nt RNA duplexes, so-called short interfering RNAs [siRNAs; (20)]. It is also possible to produce short double-stranded or hairpin RNAs within the cells, e.g. from RNA polymerase III

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expression vectors. The resulting transcripts can then be processed to active siRNAs by the endonuclease dicer (21–24). Compared with synthetic siRNAs, these DNA vectors hold the advantage that they can be stably delivered into cells and that a prolonged inhibition of targeted genes is thereby possible.

Thus, by using hairpin siRNA constructs targeting two different parts of the CyPA coding region, we obtained an efficient reduction of CyPA protein, and we succeeded in transducing these siRNA expression cassettes into CEM-SS cells using a lentiviral vector. Similar to the work using antisense U7 snRNAs against CyPA, this resulted in an impaired ability of the cells to sustain HIV-1 replication. Taken together, these results demonstrate the feasibility of inhibiting HIV-1 multiplication through a targeted down-regulation of CyPA. This approach has the potential to become a useful new tool in the fight against HIV/AIDS.

MATERIALS AND METHODS

Plasmid constructs

U7 snRNA constructs. The sequence complementary to histone pre-mRNA of plasmid U7 Sm OPT (25) was replaced by two tandem antisense sequences directed against the 3′ and 5′ splice sites flanking a particular CyPA exon, respectively (Figure 1A and B) using a previously described PCR mutagenesis strategy (17). For insertion into the single ClaI site upstream of the CMV promoter in the lentiviral vector pHR'SIN18 (26), the U7 snRNA cassette including promoter and 3′ sequences was amplified with mutagenic primers containing SfuI and ClaI restriction sites and digested by these enzymes. The insertion orientation was designated as forward (F) or reverse (R) relative to the orientation of the GFP gene. LV-glob is a control vector containing the U7/3′+5′654 snRNA cassette that targets an aberrant exon of the β-globin gene in certain forms of β-thalassemia (17).

siRNA constructs. Based on the siRNA target finder and design tool (http://www.ambion.com) and criteria suggested by Brummelkamp and coworkers (21), antisense sequences targeting nucleotides 276 to 294 (siRNA 276) or 344 to 362 (siRNA 344) of human CyPA mRNA were cloned as double-stranded DNA oligonucleotides between BglII and HindIII sites in the pSuper vector (21). The loop sequence was altered from TTCAAGAGA to TTCATATGA to introduce an NdeI restriction site (underlined), and this did not reduce the inhibitory activity of the siRNAs (data not shown). The lentiviral vector pHR'SIN18 (26) was modified by inserting a double-stranded linker containing SmaI and SalI sites in the ClaI site. The H1 cassettes carrying the CyPA siRNAs were then digested with SmaI/SalI and ligated into this pHR'SIN18-SmaI/SalI vector. A siRNA expression cassette targeting a

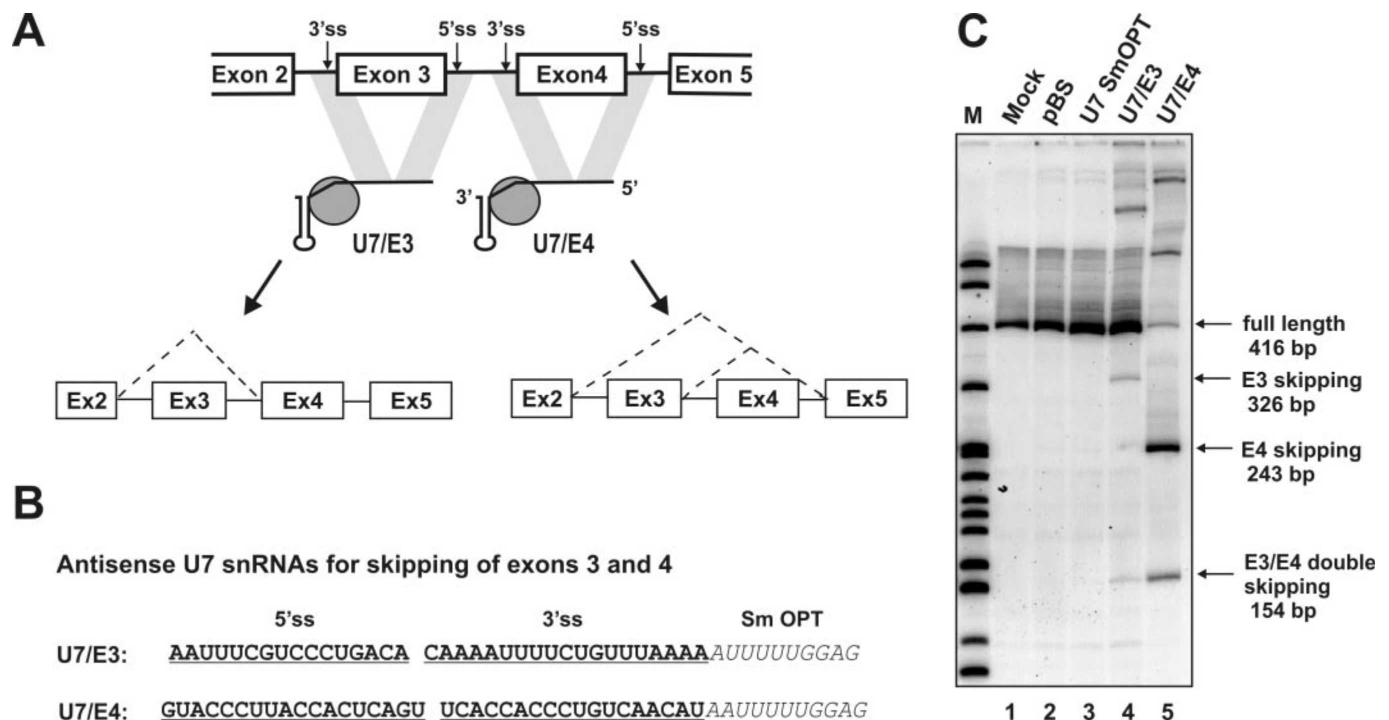

Figure 1. CyPA exon skipping induced by transient expression of antisense U7 snRNAs targeting exons 3 or 4. (**A**) Schematic drawing of modified U7 snRNAs targeting the 5′ and 3′ splice sites of exons 3 and 4 of CyPA pre-mRNA. The proteins forming the snRNP particle are depicted as a grey sphere. The resulting splicing patterns of CyPA mRNA are also indicated. (**B**) Sequences of antisense U7/E3 and U7/E4 snRNAs used to induce the skipping of CyPA exons 3 and 4, respectively. The antisense sequences targeting the 3′ and 5′ splice sites (ss) are shown in bold and underlined. The Sm OPT sequence is in italic. The 3′ terminal hairpin of the U7 snRNAs is not shown. (**C**) RT–PCR analysis of CyPA mRNA splicing in transiently transfected HeLa cells. Lane: 1, mock-transfected HeLa cells; lane 2, cells transfected with pBlueScript; lane 3, cells transfected with U7 Sm OPT, i.e. the precursor of the two anti CyPA plasmids that encodes a U7 snRNA derivative complementary to histone pre-mRNA, but which is unable to activate histone RNA 3′ end processing (25); lanes 4 and 5, cells transfected with U7/E3 and U7/E4, respectively; M, size marker (HpaII-digested, end-labelled pBR322). The sizes of PCR bands representing full length and exon-skipped mRNAs are indicated on the right.

TCRβ minigene mRNA (M. Bühler, F. Mohn and O. Mühlemann, submitted for publication) was cloned into pHR'SIN18-SmaI/SalI by the same strategy.

All clones were verified by DNA sequencing. Details of the constructs are available on request.

Cell culture, transfection and lentiviral vector transduction

293T and HeLa cells were grown in Dulbecco MEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in an incubator containing 5% CO_2. The human T-cell lines Jurkat and CEM-SS were propagated in RPMI 1640 medium containing 10% fetal calf serum and antibiotics as above. For transient transfection, 2.5×10^5 HeLa cells/well were seeded in a 6-well plate. Transfection using LipofectAmine (Invitrogen) was then carried out as described (17).

For lentiviral vector production, 6×10^6 to 6.5×10^6 293T cells were seeded in 100 mm culture dishes. After 8–16 h, 5 μg of pVSV-G, 10 μg of the packaging plasmid Δ8.91 and 15 μg of transfer vector were co-transfected by the calcium phosphate method (27). Media were changed after 12 h. Viral supernatants (10 ml) were harvested 60 h post-transfection, filtered through a 0.45 μm filter and concentrated by centrifugation at 25 000 r.p.m., 4°C for 90 min (Kontron TGA-65 ultracentrifuge, TST 28 rotor). Viral pellets were resuspended in 1 ml fresh RPMI 1640 for transduction.

For transduction, 1×10^5 HeLa cells or 5×10^5 Jurkat or CEM-SS cells were seeded per well of a 6-well plate and infected after 24 h. HeLa cells were incubated with 1 ml of unconcentrated virus containing 4 μg/ml polybrene. Jurkat and CEM-SS cells were centrifuged and resuspended in 1 ml concentrated virus containing 4 μg/ml polybrene, the suspension was centrifuged at 1500 g (Kontron TGA-6 centrifuge), 4°C for 2.5 h. Transduced cells were maintained in growth medium for 7–10 days before FACS sorting.

RNA analyses

Total cell RNA was isolated using Tri-Reagent kits (MRC, Cincinnati, OH). To detect CyPA mRNA, 250 ng total RNA was analysed by RT–PCR using rTth DNA polymerase (Applied Biosystems) for 31 cycles (94°C/60°C, 1 min each) with forward and reverse primers corresponding to sequences in exon 1 (CACCGTGTTCTTCGACATTG) and 5 (CCATGGCCTCCACAATATTC), respectively. The RT–PCR products were analysed on 6% non-denaturing polyacrylamide gels. RNase protection analysis of U7 snRNA was performed as described in (17). Dried gels were exposed to phosphor storage screens (Molecular Dynamics) and analysed with the AIDA program (version 2.31, Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Western blotting

Cells were lysed in SDS sample buffer and the lysates separated by 12% SDS–PAGE and transferred to nitrocellulose membranes overnight. Polyclonal rabbit anti-human CyPA (a gift of Dr U. Vonschwedler, University of Utah) or anti-actin (Sigma) were used as the first antibodies, anti-rabbit horseradish peroxidase-linked antibody (Promega) as the secondary antibody. Signals were detected by ECL chemiluminescence (Amersham Pharmacia) and exposure to AGFA X-ray film.

HIV-1 replication assay

3×10^4 CEM-SS cells/well were seeded in 24-well plates and infected with culture supernatant from HUT cells constitutively producing virions of the HIV-1 strain NL4-3. At various times post-infection, 50 μl supernatant samples were harvested and stored at −20°C until analysed. Virus was lysed by addition of 1/10 volume of 5% Triton X-100; 5 μl aliquots thereof were transferred directly into a complete RT–PCR master mix (ABI-Applera), supplemented with primers and VIC-labeled ENV probe (V. Brondani and T. Klimkait, manuscript in preparation), and quantitatively assessed for the presence of HIV-specific RNA. The RT step was performed at 47°C for 30 min, followed by a hot start enzyme activation for 10 min at 95°C, and 45 amplification cycles (30 s at 95°C/1 min at 57°C). All results are plotted as real-time PCR-determined RNA copies per millilitre using a characterized pNL4-3 virus stock as a quantitation standard.

RESULTS

Antisense U7 snRNA-mediated skipping of CyPA exons 3 and 4

We have developed a method, based on antisense U7 snRNAs, to promote the exclusion of internal exons from a pre-mRNA (16–18). Especially double-target U7 snRNAs, i.e. RNAs carrying two tandem sequences complementary to sites upstream and downstream of the targeted exon, respectively, very efficiently induce exon skipping (17). Presumably, these RNAs bind both targets simultaneously thereby forming a looped structure that compromises the exon's recognition by the splicing machinery.

Here, we tried to reduce the expression of the HIV-1 host factor CyPA by this approach. The CyPA gene has 5 exons, and we designed U7 double-target constructs carrying antisense sequences against the 3′ and 5′ splice sites flanking exons 3 or 4 (Figure 1A and B). Because eliminating either of these exons (or both in combination) will disturb the CyPA reading frame, we expected the severely truncated protein to be unable to interact with CA, and perhaps even to be unstable. Moreover, the mRNA might have been subject to nonsense-mediated decay.

Initially, these U7 constructs were transiently transfected into HeLa cells, and total RNA was analysed for the presence of exons 3 and 4 by RT–PCR. With RNA from mock-transfected cells or from cells transfected with two different control plasmids, a 416 bp RT–PCR product corresponding to correctly spliced, full-length CyPA mRNA was obtained (Figure 1C, lanes 1 to 3). In contrast, transfection of the U7/E3 construct yielded an additional product of 326 bp (lane 4) that corresponded to mRNA lacking exon 3, as was confirmed by DNA sequencing. Moreover, transfection of the U7/E4 construct induced a very efficient exon skipping judging from the low remaining amount of full-length CyPA mRNA (lane 5). Interestingly, besides the expected 243 bp exon 4-skipped RT–PCR band, another product of 154 bp was also detected. Sequencing revealed that it reflected the simultaneous

skipping of exons 3 and 4. A low level of double exon skipping was also observed with the U7/E3 construct (lane 4).

To analyse the effects of these U7 constructs on CyPA pre-mRNA splicing in T-cells in a persistent state, we used a lentiviral vector for stable cell transduction. The U7/E3 and U7/E4 cassettes were cloned into the vector pHR'SIN18 (26) in both the forward (F) and reverse (R) orientation (Figure 2A). The resulting plasmids were designated as LV-E3F, LV-E3R, etc. Unfortunately, no transduction was obtained with the LV-E4R vector. Although this phenomenon was not analysed in full detail, it seems to have been due to some defect in the production of viral vector genomic RNA (data not shown).

In CEM-SS T-cells stably transduced with the other three viruses, the levels of antisense U7 snRNAs were measured by an RNase protection assay (17,25). The structure of the probe and the protected products corresponding to endogenous human U7 snRNA and to the antisense U7 snRNA transcribed from the integrated lentiviral vectors are shown schematically in Figure 2B. This analysis revealed that the cells contained 2 to 3 times more of the introduced U7 snRNAs than endogenous human U7 snRNA (Figure 2C). Moreover an RT–PCR analysis of CyPA mRNA showed that the skipping of both exons 3 and 4 was enhanced compared with the results obtained in transiently transfected HeLa cells. More than 90% of CyPA pre-mRNAs were skipped with the two types of exon 3-targeting viruses (Figure 2D, lanes 4 and 5), and full-length CyPA mRNA was almost undetectable in cells transduced by LV-E4F (lane 6). In contrast, control cells transduced by the empty pHR'SIN18 vector (LV; lane 2) or by LV-glob (17), carrying an unrelated double-target U7 snRNA cassette

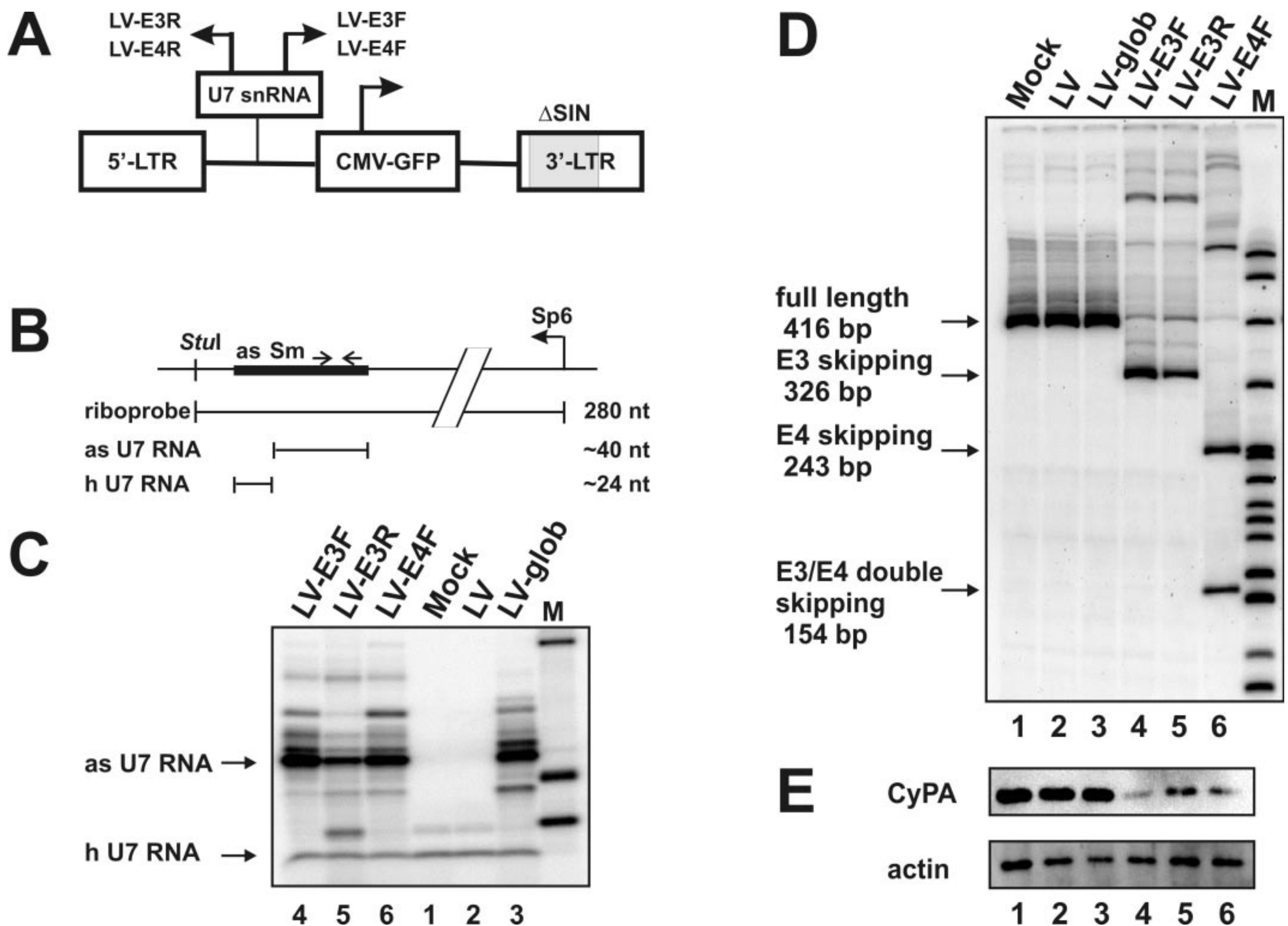

Figure 2. CyPA exon skipping and reduced CyPA protein synthesis in stably transduced CEM-SS T-cells. (**A**) Insertion of antisense U7 snRNA cassettes into the pHR'SIN18 lentiviral vector (26). The two orientations of U7 gene inserts are designated as 'F' (forward) and 'R' (reverse), relative to GFP transcription. (**B**) Structure of riboprobe used for RNase protection assay in (**C**) and of the resulting protected bands. A 280 nt riboprobe was obtained by run-off transcription with SP6 RNA polymerase to the StuI site of U7 Sm OPT (17,25). Antisense (as) U7 snRNAs expressed from the stably transduced cells are complementary to this probe over ~40 nt including the Sm OPT sequence and the 3′ terminal hairpin; endogenous human (h) U7 snRNA differs in the Sm binding site and the 3′ hairpin leaving a protected fragment of ~24 nt that can be used to monitor total RNA recovery. (C) RNase protection assay of total RNAs from stably transduced CEM-SS cells. Note that the lane order is different from that used in panels (D) and (E). (**D**) RT–PCR analysis of CyPA mRNA splicing in stably transduced cells. (**E**) Analysis of CyPA protein by western blotting probed with polyclonal anti-CyPA antibody. Polyclonal anti-actin antibody was used as loading control. Lane: 1, untransduced cells (Mock); lanes 2 and 3, cells transduced by empty pHR'SIN18 vector (LV) and by LV-glob (17) encoding a double-target U7 snRNA that targets aberrant splice sites in β-globin pre-mRNA caused by the IVS2-654 β-thalassemic mutation; lanes 4 and 5, cells transduced by LV-E3F and LV-E3R; lane 6, cells transduced by LV-E4F. Relevant bands are indicated on the left of each panel.

from a β-thalassemia system (lane 3), did not yield any skipped products, indicating that the effects of these antisense U7 snRNAs were sequence specific. Finally, CyPA protein levels were assayed by western blotting with anti-CyPA antibody. Compared to a β-actin control, the level of CyPA protein was significantly reduced in the three cell lines displaying efficient exon skipping due to the antisense U7 snRNAs (Figure 2E). Very similar results were also obtained in HeLa and Jurkat cells transduced with the LV-E3F, LV-E3R, LV-E4F vectors (data not shown).

Inhibition of CyPA expression by siRNAs

As a second approach to reduce the levels of CyPA mRNA and protein and thus to affect the cells' ability to sustain HIV-1 multiplication, we used an existing system for the production of hairpin RNAs that can be processed into siRNAs in mammalian cells (21). Based on established criteria (see Materials and Methods), two sequences corresponding to nucleotides 265 to 283 and 330 to 348 of CyPA mRNA, respectively, were chosen as potential targets (Figure 3A).

In transient transfections of HeLa cells, both of these siRNAs strongly reduced CyPA protein levels as indicated by western blot (Figure 3B). However, to achieve a prolonged inhibition, long-term and stable expression of siRNA is required. Similar to the strategy used for delivering U7 snRNA constructs, the two CyPA siRNA constructs were therefore introduced into the same lentiviral vector, upstream of the CMV promoter driving the GFP gene. However, because a directional cloning strategy was chosen, they were systematically inserted in the reverse orientation relative to the GFP gene (Figure 3C).

HeLa and CEM-SS cells were then transduced with the resulting LV-siRNA vectors and the cells were sorted based on GFP expression. Western Blots revealed that CyPA was almost completely eliminated by the expression of anti-CyPA siRNAs in both HeLa (data not shown) and CEM-SS cells (Figure 3E). In agreement with this, a semi-quantitative

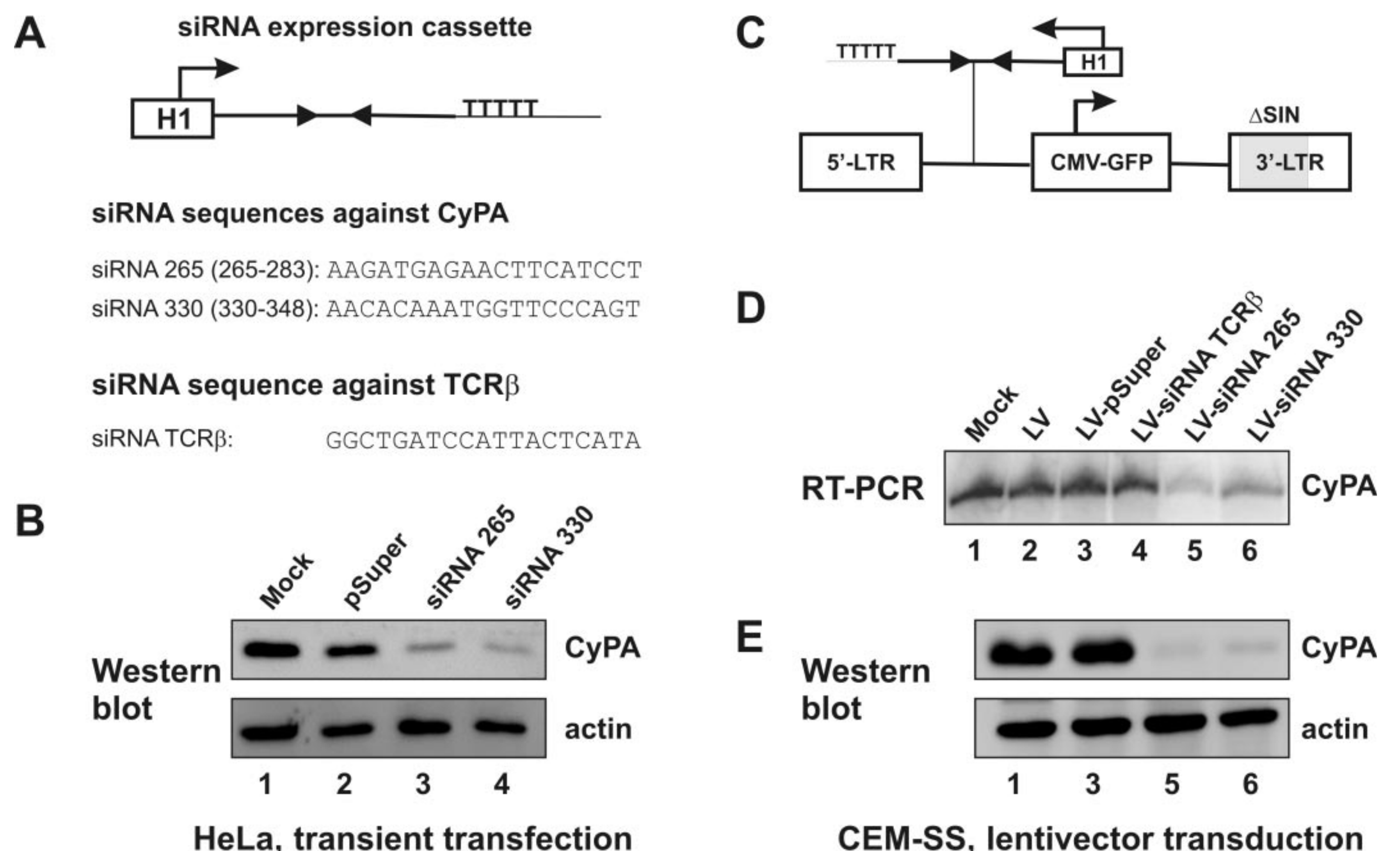

Figure 3. siRNA-mediated inhibition of CyPA expression. (**A**) Schematic diagram of the pSuper plasmid for expression of hairpin siRNAs (21). A hairpin siRNA precursor is expressed under the control of the human H1 promoter. The sequences of the siRNA targets in CyPA and TCRβ mRNAs are indicated below. For the CyPA targets, the positions from the start of the mRNA are also indicated. (**B**) Western blot analysis of CyPA protein in transiently transfected HeLa cells carried out as in Figure 2E. Lane: 1, mock-transfected cells; lane 2, cells transfected by pSuper vector; lanes 3 and 4, cells expressing anti-CyPA siRNA 265 and siRNA 330, respectively. (**C**) Schematic representation of the lentiviral vectors expressing various siRNAs. The H1-siRNA cassettes were inserted into a modified pHR'SIN18 lentiviral vector [(26); see Materials and Methods], upstream of the CMV–GFP selection marker, but in the reverse orientation respective to the GFP gene. (**D**) RT–PCR analysis of CyPA mRNA in stably transduced CEM-SS cells. The analysis was performed as in Figure 2D, except that the amplification was reduced to 25 cycles so that the signals reflect mRNA abundance semi-quantitatively (37). Only the band corresponding to full-length CyPA mRNA is shown. Lane: 1, untreated cells; lane 2, cells transduced by the empty pHR'SIN18 lentiviral vector; lane 3, cells transduced by pHR'SIN18 containing the empty pSuper expression cassette; lanes 4 to 6, cells stably transduced by pHR'SIN18-derived vectors encoding the indicated siRNAs. (**E**) Western blot analysis of CyPA protein in stably transduced CEM-SS cells. Lane numbers and cell samples correspond to those used in (D).

RT–PCR analysis showed a strong reduction in CyPA mRNA in CEM-SS cells expressing anti-CyPA siRNA 265 or siRNA 330 (Figure 3D, lanes 5 and 6, respectively). In contrast, no mRNA reduction was observed in cells transduced by lentiviral vectors containing either no siRNA cassette (LV; lane 2), an empty pSuper cassette (lane 3), or a cassette that encodes an siRNA targeting a TCRβ minigene (lane 4). Importantly, since the genes encoding the anti-CyPA siRNAs have been integrated into the genome of the targeted cells, elimination of CyPA expression will be maintained for a prolonged time, resulting in CyPA-deficient cell lines.

Delayed HIV-1 replication in CEM-SS T-cells showing reduced CyPA protein levels

Finally, we analysed whether HIV-1 replication was affected in the CEM-SS cells that showed reduced CyPA protein levels due to the expression of antisense U7 snRNAs or siRNAs. For this purpose, the replication of the HIV-1 clone pNL4-3 was followed in the stably transduced CEM-SS cells over a period of 14 days. HIV-1 multiplication in untreated CEM-SS cells initiated between days 4 to 7 post-infection, depending on the multiplicity of infection used, and peaked around day 10 (Figure 4, filled circles). Very similar multiplication kinetics were observed in CEM-SS cells transduced with the pHR'SIN18-derived vector encoding TCRβ-specific siRNA (Figure 4A and B, filled triangles). Most importantly, however, the two cell lines expressing siRNAs 265 (empty circles) and 330 (empty triangles) showed a strongly reduced viral multiplication, at both the lower (Figure 4A) and the higher (Figure 4B) multiplicity of infection. This reduction correlated with a delayed and much reduced appearance of virus-induced cell fusion events.

HIV-1 multiplication was also analysed in the CEM-SS cells transduced with the U7-containing lentiviral vectors. HIV-1 multiplication was slightly reduced in CEM-SS cells transduced with LV-glob encoding a double-target U7 snRNA that targets β-globin pre-mRNA (Figure 4C and D, filled squares). However, all three CyPA-specific vectors, LV-E3F (empty squares), LV-E4F (empty diamonds) as well as LV-E3R (data not shown), caused a more strongly delayed and reduced replication of HIV-1. In keeping with the somewhat higher residual CyPA levels detected by western blotting (see above), the effect on viral multiplication was less pronounced for these antisense U7 snRNAs than for the siRNAs targeting CyPA. Moreover, when the cells were infected at the higher multiplicity, the cumulative virus production eventually reached a similar level as seen for the cells transduced with LV-glob (Figure 4D). In summary, however, both strategies used here were effective in inhibiting HIV-1 multiplication.

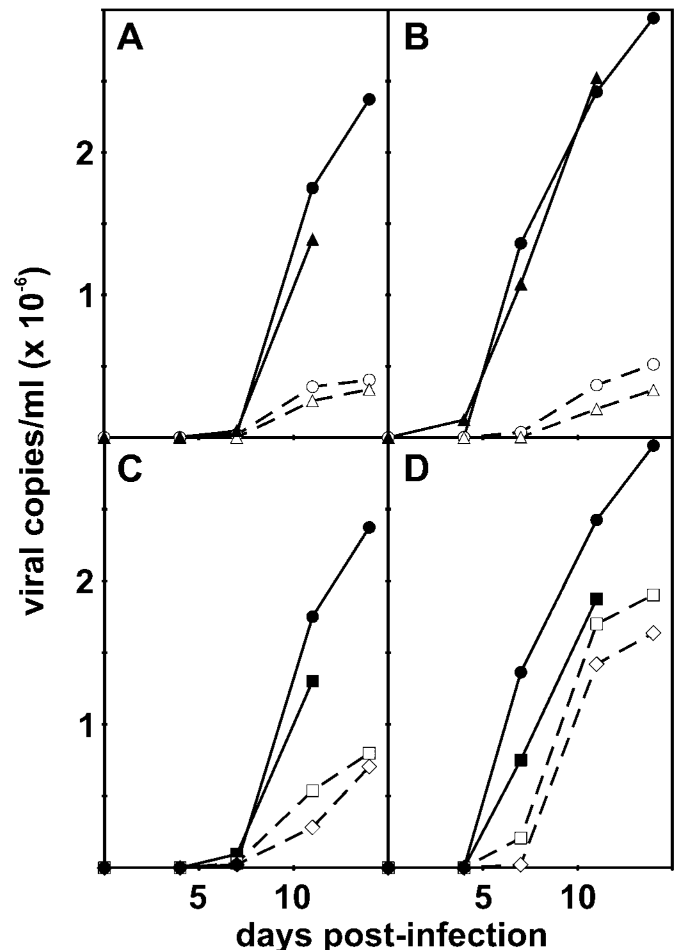

Figure 4. Reduced replication of wild-type HIV-1 in CEM-SS cells showing a permanent inhibition of CyPA expression. CEM-SS cells were infected with the HIV-1 clone pNL4-3 at multiplicities of 0.5 (**A** and **C**) or 1.7 (**B** and **D**) copies per cell. The number of viral genomes in culture supernatants was determined by quantitative RT–PCR analysis at days 4, 7, 11 and 14 post-infection, and cumulative values are shown. (A and B) Effects of siRNAs 265 (empty circles) and 330 (empty triangles). (C and D) Effects of U7 snRNAs targeting CyPA exons 3 (empty squares) and 4 (empty diamonds). As controls, the replication in untreated CEM-SS cells (filled circles) or cells transduced by pHR'SIN18 lentiviral vectors encoding either TCRβ-specific siRNA (filled triangles) or a double-target U7 snRNA that targets β-globin pre-mRNA (filled squares) are shown. All experimental values represent well correlating averages of independent duplicate experiments.

DISCUSSION

In this paper, we have described the successful application of two different molecular strategies, U7 snRNA-mediated modulation of splicing and RNAi, to reduce CyPA, a cellular protein that is important for HIV-1 replication. Through the use of lentiviral vectors, a prolonged reduction of CyPA could be obtained in a T-cell line, and, as a result, the ability of the cells to sustain HIV-1 multiplication was strongly reduced or at least delayed.

Because classical immunological approaches to combat HIV-1, due to inherent biological properties of the virus, are difficult to apply, alternative strategies, termed 'intracellular immunization' (28) bear greater promises. This term does not imply that the immune system should be introduced into cells, but rather that the cells' molecular make-up is modified in order to render them resistant to infection by HIV-1 or unable to sustain viral multiplication. Along these lines, a variety of strategies have been tested, mostly with the aim to interfere with the production or function of viral sequences or proteins. Only recently, with a more detailed dissection of the HIV-1 life cycle, certain cellular factors important for viral replication have also come into view as potential therapeutic or preventive targets [reviewed in (3)]. So far, however, the

practicability of such an approach has only been explored for few of these host factors.

In our work, we selected CyPA as the target, because this protein had been shown to play multiple roles in the early stages of HIV-1 replication (see Introduction). Moreover, several lines of evidence had shown that the protein is neither crucial for early development nor for cell viability (5,6). In a therapeutic setting, this is an important point to consider in order to minimize side effects caused by the treatment. This is of particular concern because this therapeutic approach, for being long-lasting, should not be targeted directly to T-cells, but rather to hematopoietic stem cells. Thus, CyPA would be reduced in all hematopoietic stem cell-derived lineages. In this respect, it is important to mention that down-regulating CyPA, unlike the application of csA, is not likely to cause immunosuppression, because the immunosuppressive activity of csA is not due to an interference with CyPA function but rather involves an interaction of the CyPA/csA complex with calcineurin, resulting in its inactivation (29,30). The balance, in a therapeutic situation, between any possible side effects and the benefit of having T-helper cells that are resistant to HIV-1 replication remains to be determined.

In previous work, antisense U7 snRNAs were used to affect pre-mRNA splicing to correct defective transcripts of genes affected by genetic disorders, such as β-thalassemia (16,17) or muscular dystrophy (18). In the present study, the aim was to interfere with CyPA splicing, and hence CyPA protein synthesis, to an extent sufficient to inhibit HIV-1 multiplication. This meant that a high efficiency of exon skipping had to be achieved. In general the amounts of full-length, correctly spliced mRNA and those of residual CyPA protein correlated well with each other, and, even in HeLa cells transiently transfected with the U7 constructs, the targeted exon was skipped in a significant fraction of CyPA mRNA (Figure 1). This fraction of mRNA displaying exon skipping was even higher in stably transduced CEM-SS cells (Figure 2) to the extent that full-length CyPA mRNA was sometimes virtually absent. This could either have been due to the fact that all cells expressed the antisense U7 snRNAs or to a more favorable ratio of U7 snRNA to CyPA pre-mRNA within the stably transduced cells. It has been shown previously that the CyPA concentration can vary among different cell lines (31,32). Moreover, in our experience, U7 snRNA expression in stably transduced cell lines is always quite high, reaching levels that are often higher than those of endogenous U7 snRNA (see Figure 2C for example).

Interestingly, with both U7 constructs, and in particular with the one targeting exon 4, a certain amount of combined skipping of exons 3 and 4 was observed. The skipping of the second non-targeted exon was most likely not due to a lack of specificity, because the splice sites flanking exons 3 and 4 differ as much or more from each other as those of other CyPA exons or exons from other genes which were unaffected by the treatment. A similar skipping of multiple exons was previously observed when antisense oligonucleotides (33) or U7 snRNAs (18) were used to induce the skipping of internal exons in dystrophin mRNA. This implies that certain groups of exons may be recognized by the splicing machinery in a concerted fashion so that they tend to be coordinately included in or excluded from the mRNA. However, the mechanism for this is unknown. In the case of the CyPA exons 3 and 4 targeted in this work, this phenomenon does not compromise an inhibition of HIV-1 replication, because skipping both exons has the same effect as skipping only exon 3, i.e. only a severely truncated protein consisting of exons 1 and 2 could be synthesized. It should be noted, however, that we have never observed such truncated proteins (data not shown), indicating that they may be subject to rapid degradation within the cells.

In addition to the U7 snRNA-based approach, we showed that CyPA gene expression can also be efficiently and permanently silenced using a DNA vector-based RNAi approach (Figure 3). The somewhat surprising fact that the hairpin siRNA expression cassette could be introduced into a lentiviral vector without strongly impairing the production of recombinant virions, greatly adds to the versatility of the RNAi system by providing it access to many cell types which are not amenable to DNA transfection by standard protocols. It is in line with the reports of others who have recently demonstrated the suitability of lentiviral vectors for stably introducing siRNA expression cassettes into cells [e.g. (34,35)].

Importantly, both types of antisense RNA strategies not only led to a down-regulation of CyPA levels, but also affected the cells' ability to support HIV-1 multiplication. However, the observed reduction and delay in replication was greater for the siRNAs than for the antisense U7 snRNAs, which is consistent with the fact that the reduction in CyPA protein obtained by the RNAi approach was somewhat stronger than that obtained by U7 snRNA-mediated exon skipping.

Interestingly, with even higher multiplicities of infection (⩾5 viral copies/cell) both types of antisense RNAs, did not completely abolish, but only delayed, HIV-1 multiplication (data not shown). This phenomenon is already apparent for the snRNAs at the higher multiplicity of infection shown (1.7 copies/cell; Figure 4D). A similar resumption of viral replication after an initial suppression was previously observed in Jurkat cells whose CyPA gene had been inactivated by targeted homologous recombination (6). The authors of that study demonstrated that the resumed viral replication was not due to the emergence of viral escape mutants. A possible explanation for this phenomenon is that the accumulation of a factor above some threshold concentration may overcome the block caused by the unavailability of CyPA. This could be related to a recently described role of CyPA in modulating HIV-1's response to host restriction factors for retro- and lentiviruses. Apparently, preventing the interaction between CyPA and viral capsid protein exposes the virus to the action of the human restriction factor Ref-1 (36). Since this factor is limiting, the observed delay in viral multiplication could reflect the time needed for the synthesis of sufficient capsid protein to titrate out Ref-1.

In summary, our experiments establish CyPA as a potential target for an anti-HIV therapeutic approach. Moreover, they demonstrate for the first time that U7 snRNA-mediated modification of pre-mRNA splicing can be applied to the inhibition of HIV-1 multiplication. Potentially, the siRNA and U7 snRNA approaches can be combined in a single transduction vector to obtain stronger and more lasting effects. Such an inhibition of CyPA function may provide an alternative or a complement to other HIV-1 gene therapies that already exist or are being developed.

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