Human Immunodeficiency Virus Type 1 Escape Is Restricted When Conserved Genome Sequences Are Targeted by RNA Interference[▽]

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RNA interference (RNAi) is a cellular mechanism in which small interfering RNAs (siRNAs) mediate sequence-specific gene silencing by cleaving the targeted mRNA. RNAi can be used as an antiviral approach to silence the human immunodeficiency virus type 1 (HIV-1) through stable expression of short-hairpin RNAs (shRNAs). We previously reported efficient HIV-1 inhibition by an shRNA against the nonessential *nef* gene but also described viral escape by mutation or deletion of the *nef* target sequence. The objective of this study was to obtain insight in the viral escape routes when essential and highly conserved sequences are targeted in the Gag, protease, integrase, and Tat-Rev regions of HIV-1. Target sequences were analyzed of more than 500 escape viruses that were selected in T cells expressing individual shRNAs. Viruses acquired single point mutations, occasionally secondary mutations, but—in contrast to what is observed with *nef*—no deletions were detected. Mutations occurred predominantly at target positions 6, 8, 9, 14, and 15, whereas none were selected at positions 1, 2, 5, 18, and 19. We also analyzed the type of mismatch in the siRNA-target RNA duplex, and G-U base pairs were frequently selected. These results provide insight into the sequence requirements for optimal RNAi inhibition. This knowledge on RNAi escape may guide the design and selection of shRNAs for the development of an effective RNAi therapy for HIV-1 infections.

Human immunodeficiency virus type 1 (HIV-1) accounts for millions of new infections and deaths each year. Even though antiviral therapy has improved enormously over the last decade with the introduction of highly active antiretroviral therapy, drug resistance and severe side effects remain a serious problem (31). Therefore, development of new antiviral therapies is warranted. Several gene therapy strategies have been proposed, with RNA interference (RNAi) as a promising recent addition to the antiviral arsenal. RNAi relies on an evolutionary conserved host cell mechanism in which double-stranded RNA triggers sequence-specific posttranscriptional gene silencing by cleavage of a fully complementary mRNA (7, 12, 14, 26, 34). The double-stranded RNA is provided as small interfering RNA (siRNA) or short hairpin RNA (shRNA) precursor that requires Dicer processing. Cleavage of the mRNA target is performed by the RNA-induced silencing complex (RISC), which is loaded with the guide strand of the siRNA. Intracellular expression of anti-HIV shRNAs in T cells can potentially inhibit HIV-1 replication (2, 4). However, as with most single drug antiviral therapies, shRNA-mediated inhibition is susceptible to viral escape (2, 4, 16, 27, 32, 38, 39, 41).

Nevertheless, RNAi is regarded as a promising strategy to combat a broad variety of viruses, especially in a combination

approach (11). Aside from HIV-1, other human pathogens that have been targeted include poliovirus; hepatitis A, B, and C viruses; enteroviruses; coxsackievirus; rhinovirus; and influenza virus (3, 8, 10, 15, 17, 20–22, 25, 29, 30, 33, 40, 42, 43). Escape from transient siRNA treatment has been described for poliovirus (9, 10), hepatitis C virus (42), and hepatitis A virus (20). These studies suggest that single point mutations can diminish or even abolish the RNAi effect. Repeated treatment with the same siRNA triggered the selection of multiple mutations in the viral target sequences, leading to increased levels of RNAi resistance (9, 10, 20, 42).

Most RNAi escape studies have been performed with HIV-1. Resistance against shRNA inhibitors can occur by selection of a single point mutation in the target or a partial or complete deletion (2, 4, 27, 32, 38, 39). Westerhout et al. performed the largest HIV-1 escape study thus far with the potent shNef inhibitor, which targets a nonessential sequence of the *nef* gene (Fig. 1A). Five of nine escape viruses acquired a (partial) deletion of the target, three viruses acquired a single or eventually two point mutations in the target, and one escape virus acquired a mutation 7 nucleotides (nt) upstream of the actual target (41). This mutation outside the target elicits escape by modification of the target RNA structure, reducing the accessibility for RISC and thus preventing cleavage. One of the mutations within the target caused a similar change in target RNA structure, thus causing a high level of RNAi resistance by combination of a sequence and structure change. In toto, two of nine escape viruses acquired an RNA structural change to become RNAi resistant.

In the present study, we tested for viral escape when essential, highly conserved HIV-1 sequences are targeted by

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FIG. 1. RNAi targets in the HIV-1 genome and the shRNA vector. (A) Targeted regions of HIV-1 are Gag, PR, IN, and Tat-Rev, all highly conserved sequences. The nonconserved Nef target from the previous study is also depicted (38). Four SupT1 cell lines were generated expressing individual shRNAs against these targets. Arrows indicate the primers used for PCR. (B) We used the third generation self inactivating (SIN) lentiviral vector JS1 for the generation of stable cell lines. Expression of the shRNA is driven by the polymerase (pol) III promoter H1.

shRNAs. Ideally, shRNA target sites would be identified for which escape is highly restricted or even impossible. A priori, deletions would seem impossible when essential HIV-1 genes are targeted because important genetic information would be lost. For this large-scale virus evolution study, we used four shRNAs that were previously identified as potent HIV-1 inhibitors (38). These shRNAs target essential sequences in Gag, protease (PR), integrase (IN), and Tat-Rev. (Fig. 1A). Multiple cultures of single-shRNA-expressing cells were infected with HIV-1. After extended culture, replicating virus emerged from which we analyzed more than 500 candidate escape sequences. A broad survey of escape mutations across all target sites enabled us to gain insight in RNAi resistance characteristics, e.g., the position of the escape mutations within the target and mismatch preferences.

MATERIALS AND METHODS

Plasmid construction. Lentiviral vectors and the infectious HIV-1 molecular clone pLAI were previously described (37, 38). The shRNAs Gag-5, Pol-1, Pol-47, and R/T-5 were renamed Gag, PR, IN, and Tat-Rev, respectively. IN-resistant HIV-1 LAI molecular clones were generated by site-directed mutagenesis (24). pLAI was digested with EcoRI, and the integrase fragment was cloned into pBSK to generate pBSK-IN. Mutations were introduced into pBSK-IN by site-directed mutagenesis and verified by sequence analysis, and the mutant fragment was subsequently cloned back into pLAI.

Lentiviral vector production and transduction. Lentiviral vector production, transduction, and generation of stable cell lines has been described previously (38). SupT1 cells were transduced with a multiplicity of infection of 0.15 and transduced cells were selected with live fluorescence-activated cell sorting.

HIV-1 infection on SupT1-shRNA cells. SupT1-shRNA and control cells (5×10^6 cells) were infected with the HIV-1 isolate LAI or IN-resistant variants; the viral input ranged from 0.1 to 0.5 ng of CA-p24. Virus replication was monitored by CA-p24 enzyme-linked immunosorbent assay and syncytium formation. When virus replication was observed after infection with HIV-1 LAI, cell-free virus was passaged to uninfected control and/or SupT1-shRNA cells, and virus replication was monitored.

Sequencing proviral target regions. Cellular DNA of the infected cells with the integrated provirus was isolated as previously described (19). Integrated proviral DNA sequences were PCR amplified with the following primer pairs (5'-3', the position within pLAI is indicated): Gag, sense (CAGACCATCAATGAGGAAGC TGCAGAAATGGGAT; position 1445) and antisense (CCCTGGCCTTCCCTTGT AGGAAAACAGATCTTCCC; position 2141); PR, sense (AGGCTAATTTTTT AGGGAAGATCTGGCCTTCC; position 2128) and antisense (GATATTTCTCA TGTCATCTTGGGCCTTATCTATCC; position 2788); IN, sense (GAAGCA

GAAGTTATCCCAGCAGAGACAGGGC; position 4567) and antisense (CCCA AGCTTCTAATCCTCATCCTGTCTACTTGCC; position 5157); and Tat-Rev, sense (ACCTTGTCTAGAATGGAGCCAGTAGATCCTAGACTAGAGCC CTG; position 4567) and antisense (GAAGCAGAAGTTATCCCAGCAGAGAC AGGGC; position 6407). The PCR products were gel purified and cloned into the pCR2.1 TOPO vector and subsequently sequenced with the T7 or M13R primers.

RESULTS

Experimental design of the HIV-1 escape study. We performed a large-scale viral escape study with different shRNA inhibitors targeting highly conserved HIV-1 sequences of 19 nt. These conserved regions were defined as having full identity with the HIV-1 LAI isolate and at least 75% of the HIV-1 genomes present in the Los Alamos National library database (38). Four potent shRNA inhibitors that target Gag, PR, IN, and Tat-Rev sequences (Fig. 1A) were selected from a previous screen (38). JS1 and JS1-shRNA lentiviral vectors, expressing the shRNA inhibitor under control of the polymerase III H1 promoter (Fig. 1B), were used to transduce the SupT1 T-cell line. We used a low multiplicity of infection (0.15) to obtain a single copy of the shRNA cassette per cell, and green fluorescent protein-positive cells were selected. When SupT1-shRNA and control SupT1 cells were infected with the HIV-1 LAI strain in short-term cultures, virus replication was observed only in control cells as judged by syncytium formation and massive cell death and CA-p24 production, confirming the potency of the selected shRNAs (results not shown).

SupT1-IN cells infected with HIV-1 initially show no sign of virus replication, whereas HIV-1 reaches peak infection after 9 days on the control cells (Fig. 2A). However, extended culturing of infected SupT1-shRNA cells can result in the selection of a replicating RNAi-resistant virus variant. After 19 days, replicating virus emerged in the SupT1-IN culture (Fig. 2A), which may represent an escape virus that is resistant to the shIN inhibitor. To test this, we collected cell-free virus at the peak of virus production and infected the SupT1-IN and control cells (Fig. 2B). The emerged virus (e-LAI) replicates equally well on both cell types. In contrast, the wild-type virus (LAI) from the control culture is selectively restricted on the SupT1-IN cells. This result confirms that the virus selected on

IN target

Culture:	G G A A G G A C C A G C A A A G C T C C T C T G G A A A G	G T G A A G G G G C A G T A G T A A T	A C A A G A T A A T A G T G A G T G A C A T A A A A G T A G T G C C A A G A A G A A A A G C
1		A A A	A A
2	T	T T T G G G G	
3		A A A A A A G	G
4		A A A A T	A
5		A A A A A	
6		T T T A	
7		A A A A A A	G
8		A A A A A A	
9		A A A A A A	
10		T C C C C A	G
11	G	A A A A A A	
12	G	A A A	
13		T T T T T T	
14		A A A A A A A A A A A	
15		A A A A A A	C
16		T T	
17		A A A A A A	G
18	T A	A A A	
19		A A A	
20		A A A	
21		A A A A A A	

FIG. 3. HIV sequence variation upon shRNA-IN escape. Clonal sequence analysis of the IN target and flanks upon viral escape on the nonsusceptible SupT1-IN cells. We listed the culture numbers of the clonal sequences. Changes in the sequence compared to wt are indicated for the target and 30 nt (5') and 46 nt (3') flanks.

FIG. 4. Replication test of mutant viruses. Control SupT1 (left) and SupT1-IN (right) cells were infected with wt HIV-1 LAI (◆) or mutant viruses. The mutant viruses contain mutations in the IN target at position G8A (□) or G15A (○) or both (△). Replication was monitored by measuring CA-p24 up to 10 or 12 days postinfection.

the target sequence are responsible for the loss of viral inhibition in the shRNA cell lines, we generated three IN mutants of the HIV-1 LAI molecular clone. We selected the two dominant mutations G8A and G15A. We also combined the two mutations, expecting increased RNAi-resistance. First, in a virus production assay we cotransfected the shIN construct with the pLAI variants. The most potent inhibition was scored on the wild-type virus (98%), followed by the G8A (74%) and G15A (72%) mutants and the double mutant, G8A-G15A (62%) (Y. Liu et al., unpublished results). This result indicates increased RNAi resistance by the additional point mutation within the target. Next, SupT1-IN and SupT1 control cells were infected with the LAI variants, and virus replication was monitored by measuring CA-p24 production (Fig. 4). All viruses replicated equally well on the control cells. In contrast, the wild-type virus was inhibited on the SupT1-IN cells, and only mutant viruses could replicate. In agreement with the cotransfection assay, the double mutant showed a subtle better replication on the SupT1-IN cells than the two single mutants. These results confirm viral escape in the SupT1-IN cells by point mutations within the target sequence and also confirms that secondary mutations can improve RNAi resistance.

Mutations in the target. For all shRNA inhibitors the primary sequence data was analyzed. As observed in Fig. 3 for IN, only scattered mutations were observed outside the target for all shRNAs, which were thus not included in our subsequent analysis shown in Fig. 5. The 19 nt positions of the target are numbered from 5' to 3'. The frequency of each type of escape mutation and its position within each target is shown. Escape mutations are likely to become dominant in the viral quasiespecies and will thus be present in multiple clonal sequences. We therefore only scored sequence changes that were observed in at least two clonal sequences, either from the same or a second culture. By doing so, we also restricted the chance of scoring false positives (e.g., spontaneous sequence variation, sequencing and/or PCR error). The number of occurrences of the indicated mutations in different cultures is listed in the middle column. In various cultures more than one type of mutant was present, consistent with the early sampling strategy.

We observed many wild-type sequences in the Gag experiments (14 of 16 cultures), with 8 cultures showing exclusively

wild type, but an additional viral passage yielded sufficient mutations to be included in the survey. For the other three shRNA inhibitors, the selection pressure is apparently stronger, since we observed only a single clone with the input wild-type target sequence for PR and IN after the first passage (in 16 and 21 cultures, respectively). This wild-type sequence was not included in the survey of Fig. 5, because we only count sequences that occur in at least two clones. Several mutations were selected repeatedly in multiple cultures. For instance, 12 of 21 IN cultures contain viruses with the G8A mutation, and 13 of 17 Tat-Rev cultures show a mutation at position 15, either C-to-U (7x) or C-to-A (6x).

Preference for silent mutations in the targets. Since all targets were selected as conserved HIV-1 genome regions that encode essential viral proteins, mutations within the target may affect the encoded amino acids (nonsilent), although silent codon changes are also possible. We therefore listed the amino acid substitutions in the last column of Fig. 5. There is indeed a preference for silent codon changes. This trend is most obvious for PR cultures, for which we score 25 silent and only 3 nonsilent escape routes. Furthermore, these three nonsilent mutations are identical and result in the amino acid substitution D30N in the PR enzyme. These results suggest that other nonsilent changes are not allowed in this PR domain.

The Tat-Rev overlap provides a special situation, since double-silent-codon changes are nearly impossible due to the overlapping reading frames. Only two possibilities exist for such a double-silent escape: A6C and A18C. In fact, no RNAi escape mutations occurred at position 18 in all cultures analyzed in the present study, and we did not observe the A6C mutation in the Tat-Rev overlap either. Interestingly, of the 40 escapes routes, 20 are silent in Tat, and only 8 are silent in Rev. This result is consistent with the importance of the encoded Tat domain and the relative unimportance of the encoded Rev domain. The Tat domain encodes the basic stretch of amino acids (48GRKKR52) that encodes the nuclear localization signal and the TAR-binding domain (5). In fact, many of the nonsilent Tat changes represent conservative amino acid changes (e.g., R49K, K50R, and K51R). These combined results strongly suggest that the virus is under pressure to maintain Tat function during shRNA escape. Selection pressure

Gag position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	16 #	a.a. changes Gag (CA-p24)					
	G	A	A	G	A	A	A	U	G	A	U	G	A	C	A	G	C	A	U	14	E	E	M	M	T	A
	-	-	G	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-	-	4	-	D	-	-	-	-
	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	5	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	3	-	-	-	-	S	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	2	-	-	-	-	I	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	1	-	-	-	-	R	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	1	-	-	-	-	-	T

Protease position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	16 #	a.a. changes Protease					
	A	C	A	G	G	A	G	C	A	G	A	U	G	A	U	A	C	A	G	0	T	G	A	D	D	T
	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-
	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	2	-	-	-	-	N	-
	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	7	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	C	-	-	-	1	-	-	-	-	N	-

IN position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21 #	a.a. changes Integrase				
	G	U	G	A	A	G	G	G	G	C	A	G	U	A	G	U	A	A	U	0	E	G	A	V	V
	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	V	-	-	-	-
	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	G	-	-	-	-
	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	A	-	-	-	-
	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	12	-	-	-	-	-
	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	5	-	-	T	-	-
	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	1	-	-	V	-	-
	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	3	-	-	-	L	-
	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	1	-	-	-	I	-
	-	-	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	1	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	7	-	-	-	I	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	1	-	-	-	-	-
	-	-	-	U	-	-	-	-	A	-	-	-	-	-	-	-	-	G	-	1	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1	V	-	T	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	1	-	-	-	I	-

Tat-Rev position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	17 #	a.a. changes Tat	a.a. changes Rev				
	A	U	G	G	C	A	G	G	A	A	G	A	A	G	C	G	G	A	G	10	G	R	K	K	R	M
	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	D	-	-	-	-	-
	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	2	-	K	-	-	-	-
	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	3	-	-	R	-	-	-
	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	1	-	-	T	-	-	-
	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	1	-	-	-	R	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	5	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	7	-	-	-	-	W	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	5	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	1	-	-	-	Q	-	-
	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	A	-	-	1	-	K	-	-	Q	-
	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	-	-	-	-	2	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	G	-	A	-	-	-	-	-	1	-	-	-	R	-	-
	-	-	-	-	-	-	-	A	-	-	-	-	-	A	-	-	-	-	-	1	-	-	-	-	-	-

FIG. 5. Escape mutations in the 19-nt targets. The four targets are shown from 5' to 3', positions 1 to 19. Each type of mutation was scored only once per culture; the number of occurrences is depicted in the middle column. Amino acid changes are shown in the right column.

at the protein level may thus influence the type of target site changes that are available for viral escape.

Positional hotspots within the target for viral escape. The escape data clearly indicate that the virus does not use all 19 positions within the target to become resistant to the shRNAs. For instance, position 15 is mutated repeatedly in the Tat-Rev culture (12x) with two different type of substitutions, and this position is also mutated in many PR and IN cultures (8x and 7x, respectively), while other positions are underrepresented or even absent. We analyzed the combined escape data for the

four shRNAs to neutralize shRNA-specific effects. A total of 113 target site mutations were scored, excluding the targets with a double mutation. We plotted the number of mutations occurring for each of the 19 target positions (Fig. 6). Within the target, two hotspot regions for RNAi escape mutations are observed. The frequently used escape positions are 6, 8, 9, 14, and 15, the latter being responsible for almost a quarter of the total number of mutations. The terminal 2 nt at both ends of the target (positions 1, 2, 18, and 19) and position 5 do not acquire any escape mutation. These results suggest that there

FIG. 6. Distribution of all escape mutations. Per target site position, we plotted all mutations observed in the four shRNA escape studies depicted in Fig. 5. Escape variants with an additional mutation in the target were excluded from this analysis.

is a clear hierarchy in the target positions with respect to RNAi escape. Both termini and the center of the target sequence are strongly underrepresented, and the domains flanking the center are overrepresented.

Type of disruption of the target-siRNA duplex. We also analyzed whether there is a preference for certain mismatches in the target-siRNA duplex of escape viruses. A priori, mismatches of bulky purine samples (G-G and A-A) could be expected to be most destructive to duplex stability and consequently result in effective RNAi resistance. In contrast, mutations that result in G-U base pairing may be the least disruptive. We tabulated the different duplex disruptions in Table 1. We ranked the type of base pairing from weak to profound duplex destabilization: Pu-Py (G-U type), Pu-Py (C-A type), Py-Py, and Pu-Pu. This analysis was performed for all single mutations of the survey presented in Fig. 5. Surprisingly, Pu-Py mismatches are overrepresented in escape viruses, despite the fact that this group includes the least disruptive G-U base pair. In contrast, the more disruptive Py-Py or Pu-Pu changes were not frequently selected. This is a puzzling result that requires further analysis.

Realizing that the mismatch types are generated by different type of mutations and considering the particular mutational bias of HIV-1 (23), we investigated whether this bias may influence the outcome. Indeed, all Pu-Py combinations are generated by transitions, which occur more frequently than transversions. Transversions are needed to generate Pu-Pu and Py-Py base pairs. In fact, the mutational bias also explains the preponderance of the C-A mismatch, since the G-to-A mutation is the most prevalent in HIV-1 (Table 1) (23). Thus, the mutational bias explains the frequent selection of mutations that cause a weak duplex destabilization.

DISCUSSION

No shRNA inhibitors were identified in this large-scale HIV-1 replication study that do not allow viral escape, despite the fact that highly conserved viral genome sequences were

targeted. However, evidence for a strong restriction of the viral escape possibilities is apparent for these shRNAs. Sequence deletion is a popular escape route when the nonessential *nef* gene is targeted (41), but no such escape was apparent for the highly conserved targets in essential genes, which is consistent with our prediction. For all shRNA inhibitors, we observed further restrictions. This is most notable for the PR shRNA, for which nonsilent changes were profoundly counterselected (only 3 of the 28 cases). Targeting of the Tat-Rev overlap sequences provided another interesting restriction pattern: nonsilent changes occurred preferentially in the Rev protein and not in Tat protein, and the few amino acid changes selected in Tat are conservative in nature. We interpret these results in terms of viral replication fitness. Many target mutations are able to provide RNAi resistance, but viral fitness can be reduced by mutation of important regulatory sequences and/or essential amino acids.

We previously described an exotic escape route for shNef in which the escape mutation occurs outside the actual target sequence (41). Resistance is mediated by a change in the structure of the target RNA, which becomes inaccessible to the RNAi machinery. We did not obtain additional examples of such an RNA structure-based escape in this large-scale evolution experiment, since all escape viruses had a mutation within the 19-nt target and no changes outside the target became fixated in the viral genome. Although mutations within the target may also affect the local RNA structure, structure-mediated escape does not seem to be a prevalent evolutionary route.

The severe restriction of viral escape with some shRNAs provides an interesting therapeutic possibility. For instance, in the PR cultures we observed a limited number of seven escape routes that use only 5 of the 19 possible target positions. This

TABLE 1. Disruption of the target RNA-siRNA duplex

Target-siRNA pairing ^a	Causative mutation	No. of occurrences ^b
Pu-Py		
G-U	A→G	28
U-G	C→U	10
Total		38
Pu-Py		
C-A	U→C	7
A-C	G→A ^c	46
Total		53
Py-Py		
U-C	G→U	3
C-U	A→C	2
U-U	A→U	11
C-C	G→C	0
Total		16
Pu-Pu		
A-G	C→A	5
G-A	U→G	0
A-A	U→A	0
G-G	C→G	1
Total		6

^a That is, a G-U base pair or mismatch (all others).
^b Only single mutations in the target are counted.
^c G→A is the most common mutation in HIV-1.

restriction is probably due to the counterselection of nonsilent codon changes, except for the G13A mutation that causes an Asp (D)-to-Asn (N) substitution at position 30 in the PR enzyme. With only seven escape routes, one could consider the use of a second generation therapy adding seven shRNAs that target these mutant sequences (36). The absence of more nonsilent mutations indicates that this PR domain is critical for enzyme function, and we expect that amino acid substitutions lead to viruses with reduced fitness. Strikingly, the same D30N substitution is selected upon treatment with the PR inhibitor Nelfinavir (28). These combined results suggest that this is one of the few possible substitutions in this PR domain, which is consistent with a severe restriction of the evolutionary possibilities. The selection of RNAi resistant, yet attenuated HIV-1 variants may be of clinical relevance, since it may result in a reduced viral load and delayed disease progression.

Although HIV-1 escapes from all shRNAs that we tested thus far, we still have approximately 20 other potent shRNA inhibitors that have not yet been tested for viral escape. We recently showed that viral escape could be delayed and even prevented by the combined action of multiple shRNA inhibitors (35, 38). Strong inhibitors such as IN and PR that exhibit a strong restriction of the possible viral escape routes are ideal shRNA candidates to be used in such a combination therapy.

Virus escape studies also provide some insight into the mechanism of RNAi, in particular the requirements for target-siRNA pairing. We observed certain hotspots and cold spots for viral escape when tabulating all escape data (Fig. 6). Most notably, no escape mutations were selected at both termini of the 19-nt target, positions 1, 2, 18, and 19 and also position 5, suggesting that these nucleotides do not significantly contribute to the efficiency of RNAi-mediated inhibition. Given the importance of the central domain (positions 3 to 17), shRNA inhibitors should perhaps be redesigned against highly conserved 15-nt stretches, thus expanding the number of potential RNAi targets. Of course, natural sequence variation is limited in these HIV-1 genome regions because we exclusively selected highly conserved targets (38). Thus, we also compared the natural sequence variation in different HIV-1 isolates from patients with our extensive set of RNAi escape mutations (not shown). For instance, we scored a lot of natural sequence variation at position 18 and 19 of the four HIV-1 targets, and yet these positions are not used for RNAi escape. This finding strengthens our conclusion that these positions cannot provide an RNAi-resistant phenotype, since they are allowed to change in nature. The situation is quite different for positions 1, 2, and 5 that were also not selected in the RNAi experiments. The same pattern is observed within the targets of patient isolates. The absence of much variation may indicate that such virus mutants will be replication impaired, which in fact could explain their absence in our experiments as well.

Moreover, several RNAi hotspots, especially at positions 3 and 6, are also hotspots of natural sequence variation in HIV-1 isolates. Our interpretation is that many alternative RNAi-resistant mutants could have been selected, but only a few maintained viral fitness, e.g., by using silent codon changes. These observations confirm the idea that viral escape is severely restricted, which is imposed by the selection of highly conserved targets in our therapeutic strategy.

Surprisingly, we observed the preferential selection of mu-

tations that cause a relatively weak disruption of the target-siRNA duplex with G-U base pairs and C-A mismatches. This result is striking and seems contradictory to the results of Du et al. (6), who reported that such mutations are generally well tolerated by the RNAi machinery. However, one should keep in mind that virus evolution consists of two independent steps: the generation of the mutation and the subsequent selection of the escape variants. The prevalence of weakly disruptive duplex changes (A-C and G-U) is likely due to the fact that these are relatively easy to generate by a transition type of mutation (A-to-G, G-to-A, U-to-C, and C-to-U). HIV-1 has a profound mutational bias, strongly preferring transitions over transversions, and we previously demonstrated that this bias can determine the outcome of HIV-1 evolution, e.g., the acquisition of drug resistance (1, 18). In fact, the most prevalent mutation is G to A, which explains the frequent selection of A-C mismatches. In addition, in our experimental setup we used only a single shRNA cassette per cell, resulting in relatively low shRNA expression that may allow weak disruptions to be RNAi resistant. Thus, the result of this virus evolution study is not inconsistent with the Du study, since our study represents a more complex and perhaps more sensitive biological system.

A previous study reported enhanced RNAi activity by incorporation of weak G-U base pairs in the target-siRNA duplex (13). The frequent selection of these G-U base pairs in this evolution study does not support this idea, although more detailed studies are required to formally exclude this enhancement proposal.

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