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Specific inhibition of human immunodeficiency virus type 1 replication by antisense oligonucleotides: An *in vitro* model for treatment

(escape mutants/therapeutic avoidance)

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ABSTRACT **We have developed a culture system, simulating *in vivo* conditions of human immunodeficiency virus type 1 (HIV-1) infection, to evaluate the long-term efficacy of antisense oligonucleotide treatment. Five oligonucleotide phosphorothioates (28-mers), complementary to different regions of HIV-1 RNA, blocked replication of the virus in a sequence-specific manner at 1 μM concentration. Variations in antiviral activity were seen among the different oligonucleotides, revealing an effect of target selection. Mismatched or random oligonucleotide phosphorothioates delayed, but did not completely inhibit, HIV-1 replication. In the case of inhibition by a splice-acceptor-site antisense oligodeoxynucleotide, a breakthrough phenomenon occurred after 25 days of treatment, suggesting the development of an "escape mutant." This result did not occur when the inhibitory oligodeoxynucleotides were complementary to the primary-sequence areas of the rev-responsive element and *rev-1* genes. Sequential treatment of HIV-1-infected cells with a combination of different antisense oligonucleotides, each administered once, also prevented the development of escape mutants. Our results suggest that chemotherapy based on specifically targeted antisense-oligonucleotide phosphorothioates may be an effective method for reducing the viral burden in HIV-1-infected individuals at clinically achievable oligonucleotide concentrations.**

One rationale for antiviral chemotherapy is based on the use of antisense oligonucleotides to inhibit specifically the expression of human immunodeficiency virus type 1 (HIV-1) (1) or other viruses (2). Unmodified phosphodiester oligodeoxynucleotides, complementary to HIV-1 RNA, have been demonstrated to inhibit viral replication in early infected cells (3, 4) but not to inhibit viral replication in chronically infected cells (5), mainly because of the nuclease susceptibility of these oligodeoxynucleotides (6). Therefore, chemically modified nuclease-resistant analogs have been developed and studied for their effectiveness in inhibiting HIV-1 replication in tissue cultures (7–9). Phosphorothioate-modified oligomers inhibit HIV-1 replication in both acute infection (5) (when virus is added to an uninfected susceptible-cell line) and in chronically infected cell lines (8, 10–12), but even mismatched oligomers have some inhibitory activities in acutely infected cells. At low concentrations, these control oligomers are, however, less effective than the complementary oligomers.

MATERIALS AND METHODS

Synthesis of the Oligodeoxynucleotide Phosphorothioates. Phosphorothioate-modified oligodeoxynucleotides were synthesized by using H-phosphonate chemistry on an automated synthesizer (Millipore model 8700) on a 5- to 10-mmol scale. After assembly of the required sequence, the controlled-pore glass-bound oligonucleotide H-phosphonate was oxidized with sulfur in pyridine/triethylamine/carbon disulfide to generate phosphorothioate linkages. The deprotection was completed in concentrated ammonia at 40°C for 48 hr. The oligonucleotides were purified by preparative reverse-phase chromatography and then ion-exchange chromatography. Finally, purified oligonucleotides were dialyzed against water and lyophilized. Oligonucleotide phosphorothioates were checked for their purity by HPLC and PAGE (5).

Antisense and Control Oligonucleotides. To study the inhibition of HIV-1 replication, oligodeoxynucleotide phosphorothioates 28 bases long were chosen because specificity has been shown to be increased by length of the oligomers used at equivalent concentrations (11). Five different target sequences (Fig. 1) in the HIV-1 genome were selected. Because *tat* and *rev* can trans-activate HIV-1 gene expression and are essential for virus replication, two oligomers, rev-1 (10) and rev-2, complementary to these overlapping reading frames were evaluated. Rev regulates HIV gene expression through interaction with the Rev-responsive element (RRE). Therefore the RRE oligomer was directed against the RRE overlapping the known Rev-binding site (14, 15). The RRE oligomer was chosen after screening a number of potential anti-RRE oligonucleotides. In addition to blocking the Rev–RRE interaction, RRE could potentially interfere with translation of the viral envelope gene. Another oligomer, designated gag, was directed against the mRNA of that structural gene. Oligonucleotide SA is complementary to the major splice-acceptor site of the first coding exon of the *tat* gene (16), which is also located in the open reading frame of the nonessential *vpr* gene. To determine specificity of the antisense oligomers, the biological effect should be compared to the same-size oligomer that is not complementary to any known cellular or viral genes. We have chosen three control "nonspecific" types of oligonucleotides, of which the "Random" sequence is theoretically the best. The Random sequence was synthesized as a degenerate oligonucleotide, by coupling a mixture of 4 nucleotides at each stage (theoretically it contains $4^{28} = 7.2 \times 10^{16}$ sequences) and thus measures the extent of sequence-nonspecific inhibition. "Mismatch" has the SA sequence with 5 bases altered (Fig. 1, underlined bases) and serves as a type of control for

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RRE, Rev-responsive element; RT, reverse transcriptase; SA, oligonucleotide complementary to the major splice-acceptor site of the first coding exon of *tat*.

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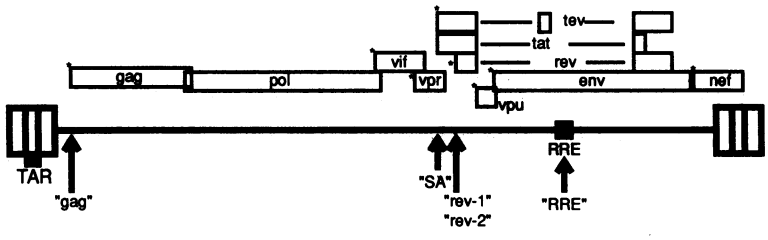

"SA"	5'-	GTC	GAC	ACC	CAA	TTC	TGA	AAA	TGG	ATA	A	5763-5790
"Mismatch"	5'-	GTC	CAC	ACG	CAA	TTG	TGA	TAA	TGC	ATA	A	———
"rev-1"	5'-	TCG	TCG	CTG	TCT	CCG	CTT	CTT	CCT	GCC	A	5970-5997
"rev-2"	5'-	CTG	TCT	CCG	CTT	CTT	CCT	GCC	ATA	GGA	G	5976-6003
"RRE"	5'-	CCC	ATA	GTG	CTT	CCT	GCT	GCT	CCC	AAG	A	7788-7815
"gag"	5'-	CGC	TCT	CGC	ACC	CAT	CTC	TCT	CCT	TCT	A	776-802

FIG. 1. Sequences of antisense oligonucleotide phosphorothioates used and targeted genes in HIV-1 genome (13).

SA-specific inhibition. Areas of potential hybridization in this mismatched oligomer still remain, however, with a likelihood of activation of RNase H. C28 is a phosphorothioate oligodeoxycytidine, S-$(dC)_{28}$. The latter homooligomer has a significant antiviral effect (8, 10), has the potential for segments of hybridization, and also introduces cytotoxicity.

Short-Term Experiment for Inhibition of HIV-1 Replication. MOLT-3 cells (5×10^5 per ml) were infected with HIV-1 type III_B. After 2 hr, cells were washed and treated with oligonucleotide phosphorothioates at 1 μM concentration. Cells were incubated for 4 days, and then virus expression was measured.

Long-Term Experiment for Inhibition of HIV-1 Replication. MOLT-3 cells (5×10^5 per ml) were infected with HIV-1 type III_B. After 2 hr, cells were washed and treated with oligonucleotide phosphorothioates at 1 μM concentration. Cells were split to 5×10^5 cells per ml every 3 or 4 days and treated with drugs at a concentration of 1 μM.

Detection of HIV-1 Expression and Toxicity. Virus replication was monitored at the cellular level by syncytia formation and p24 membrane expression (7) and in the supernatants by p24 antigen-capture assay (DuPont) and reverse transcriptase (RT) assay (17). Cell viability was determined by trypan blue exclusion.

RESULTS AND DISCUSSION

Sequence-Specific Inhibition of HIV-1 Replication. Studies of the mechanism and efficiency of antisense oligonucleotides in inhibiting viral replication can be approached effectively in an *in vitro* system that resembles the process of HIV-1 infection *in vivo*. In HIV-1-infected people, only a small percentage of the $CD4^+$ cells are infected and producing virus. *In vitro* chronically infected cells do not parallel the *in vivo* conditions because these cells are $CD4^-$ and consequently reinfection cannot occur. A better model for drug studies may be an acute, low multiplicity of infection, where only some fraction of the cell population harbors virus, while other cells are uninfected and $CD4^+$. We have developed cell culture conditions to approach those *in vivo* conditions. This system has similarities to a retroviral vector, one used for studies of HIV-1 inhibition (18). The effect of antisense oligonucleotides on HIV-1 replication was tested in a $CD4^+$ T-cell line (MOLT-3) infected with low multiplicity of infection of HIV-1 type III_B. After 2 hr of infection, the cells were washed and treated with oligonucleotides. The initial infection is characterized by 3% positivity for p24 membrane expression after 4 days in untreated cells. Therefore, most cells remain $CD4^+$ and, thus, susceptible to reinfection during the treatment period. Extending the treatment period over weeks rather than days simulates a treatment schedule that could be given to an HIV-1-infected individual.

The amount of drug used for long-term studies was determined, based on the minimal concentration that inhibited viral infection >60% in a 4-day study. Short-term inhibition by different oligomers (Fig. 2*A*) demonstrated that all compounds other than Random inhibited HIV-1 replication >60% compared with control (cells infected without drug). No more than 26% difference in antiviral activity was found among the complementary and "control" oligomers other than the Random control in our short-term assay. Twenty-five days after infection, however, high levels of virus replication were detected in cultures treated with both the Random and the Mismatch oligomers, demonstrating that these control oligomers failed to inhibit HIV-1 replication (Figs. 2*B* and 3*A*). Because these controls were at the peak of the acute phase of the infection at day 25, this time was chosen to evaluate the specificity of inhibition. Inhibition of HIV-1 replication by all complementary oligonucleotides was >99.8% (detected by a quantitative p24 ELISA assay) compared with the Random oligomer. Less than 1% of cells treated with the sequence-specific antisense oligomers expressed detectable p24 antigen on the surface, and only 3% of the SA-treated cells showed syncytia formation (Fig. 2*B*). In these cultures the numbers of viable cells resembled those in the uninfected control, suggesting that long-term oligonucleotide phosphorothioate treatment is not toxic to cells. The acute phase of HIV-1 infection in the control is characterized by extensive syncytia formation (>75%), low membrane-antigen expression, and high virus levels in the supernatants (p24 >1 μg per million cells). Cells that survive enter into a chronically infected phase (19) characterized by the absence of a CD4 receptor at the cell surface. Because CD4 is the main receptor for HIV-1 infection of T cells, reinfection does not occur, and production of virus declines. Therefore, a chronically infected culture produces at least 100 times less virus than cells in the acute phase and does not present a cytopathic effect.

At day 25, an aliquot of the cells was split without addition of the antisense oligomers. After four passages without drugs, all cultures entered an acute phase of infection as described above. Fig. 2*C* demonstrates that recovery of virus replication was delayed depending on the oligomers used for treatment during the first 25 days. The earliest recovery of the virus was in the SA-treated cells at day 32. This early reappearance might be expected, considering that SA is not directed against the open reading frame of an essential gene. At day 35 gag- and RRE-treated cells converted to an acute phase of infection, consistent with specific inhibition at the late phase of the virus replication. The rev-2-treated, followed by rev-1-treated, were the last cells to revert into an acute phase of infection. These oligomers presumably inhibit the early phase of virus replication by interfering with activity of the regulatory genes. This delay in recovery is consistent with the proposal that a threshold level of Rev protein is

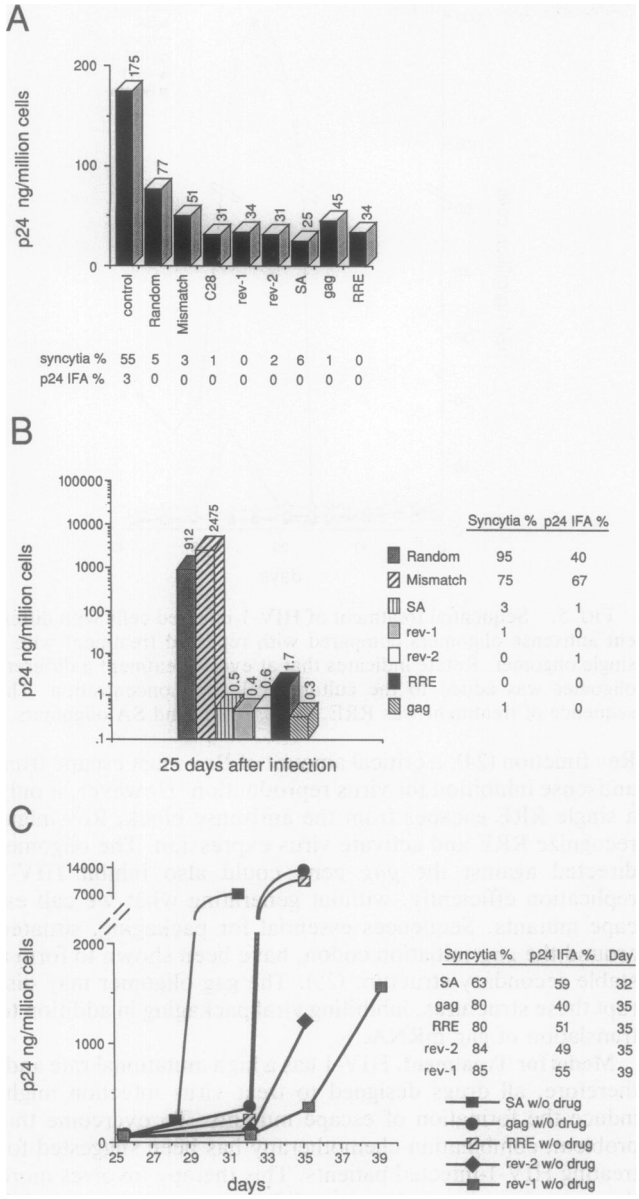

FIG. 2. Antiviral activity of oligonucleotide phosphorothioates. (*A*) Antiviral activity of oligomers in short-term assay. (*B*) Specific inhibition of HIV-1 replication by complementary oligomers in long-term assay. IFA, immunofluorescence assay. (*C*) HIV-1 replication after drug treatment. After 25 days of treatment, an aliquot of cells was split and grown without (w/o) drugs.

required to get full expression of single or unspliced viral mRNA, consequently permitting virus replication (20, 21).

Antiviral Activity Depends on Targeted Sequences. The test system used for these experiments allowed time for the virus to generate "escape mutants," a phenomenon not previously observed in studies based on short-term cultures (quotation marks are used because other explanations for this effect are possible, although this seems a probable one). Fig. 3*A* shows that 32 days after infection, a population of virus escaped from the inhibitory effect of the SA oligomer. At this time, 66% of this culture showed syncytia, and 20% of cells expressed the p24 membrane antigen, although this percentage was still significantly less virus compared with cultures during the acute phase of infection when treated with Random or Mismatch oligomers (Fig. 2*B*). Inhibition of HIV-1 replication in cultures treated by rev-1, rev-2, gag, and RRE oligomers was >98% (detected by p24 antigen-capture assay of the supernatants), compared with the SA-treated culture

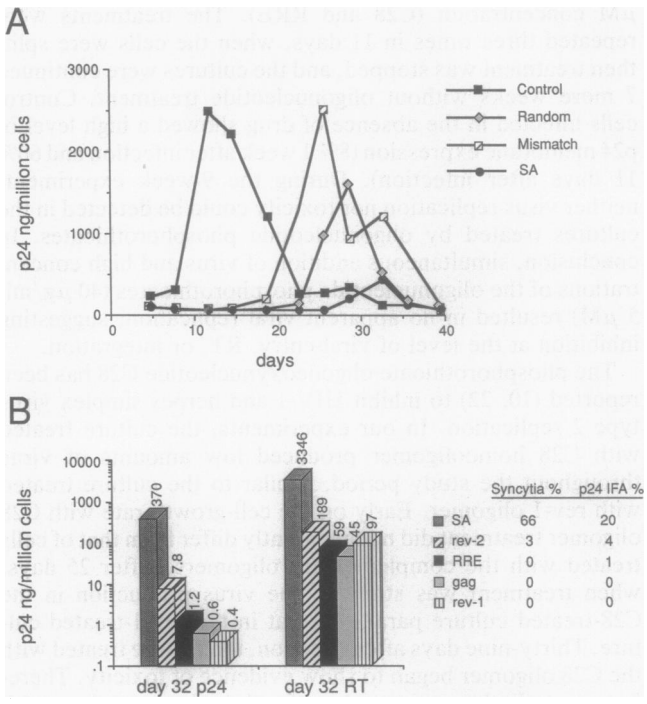

FIG. 3. Generation of escape mutants after SA treatment and comparison of different antisense oligonucleotides. (*A*) Escape of SA oligomer compared with Random, Mismatch, and control (no drug treatment). (*B*) Differences in antiviral activity between the complementary oligonucleotides. IFA, immunofluorescence assay.

(Fig. 3*B*). The RT assay is not quantitative at the lower range; however the RT assay results supported the p24 antigen-capture assays. These results suggest that there was an escape from the inhibitory effect of SA oligomer not apparent early in treatment. Expression of the Tat protein can result from alternatively spliced messages using an upstream splice acceptor for the second exon (16). A shift in use of an alternative splice site or a mutation in the targeted sequence might have occurred during the SA treatment. Because HIV-1 type III_B contains a defective *vpr* gene, we do not know that SA oligomer alters the function of this protein. Interestingly, rev-1 oligomer inhibited 20-fold better than rev-2 oligomer (see quantitative results on the p24 antigen-capture assay, which is supported by syncytia, RT, and membrane expression), even though they contain 22 nucleotides of overlapping sequence. This result indicates that minor shifts in the targeted sequences can cause variances in efficacy of these oligomers. Notable differences in antiviral activities of the complementary oligomers were detected at day 32; the most efficient oligomers after long-term treatment were rev-1 and gag followed by RRE and rev-2. These differences were maintained for as long as the cultures were monitored.

Non-Sequence-Specific Antiviral Activity of Oligonucleotide Phosphorothioates. Fig. 3*A* shows early development of high levels of viral replication in controls. Similar development of high levels, ≈11 days later, occurred in cultures treated with 1 μM concentration of Random or mismatched oligodeoxynucleotide phosphorothioates, as described earlier for long-term experiments. This result suggests that oligonucleotide phosphorothioates may have a sequence-nonspecific antiviral effect, which delays, but does not effectively inhibit, virus replication. Since as few as 2–4 duplex bases in mixed hybrid complementarity can activate RNase H, however, it is uncertain as to whether the term *random* represents complete lack of sequence specificity.

MOLT-3 cells were infected with HIV-1 by simultaneous addition of virus and oligonucleotide phosphorothioates at 5

μM concentration (C28 and RRE). The treatments were repeated three times in 11 days, when the cells were split; then treatment was stopped, and the cultures were continued 7 more weeks without oligonucleotide treatment. Control cells infected in the absence of drug showed a high level of p24 membrane expression (8% 1 week after infection and 60% 11 days after infection). During the 9-week experiment, neither virus replication nor toxicity could be detected in the cultures treated by oligonucleotide phosphorothioates. In conclusion, simultaneous addition of virus and high concentrations of the oligonucleotide phosphorothioates (40 μg/ml; 5 μM) resulted in no apparent viral replication, suggesting inhibition at the level of viral entry, RT, or integration.

The phosphorothioate oligodeoxynucleotide C28 has been reported (10, 22) to inhibit HIV-1 and herpes simplex virus type 2 replication. In our experiments, the culture treated with C28 homooligomer produced low amounts of virus throughout the study period, similar to the culture treated with rev-1 oligomer. Early on the cell-growth rate with C28 oligomer treatment did not apparently differ from that of cells treated with the complementary oligomers. After 25 days, when treatment was stopped, the virus production in the C28-treated culture paralleled that in the rev-1-treated culture. Thirty-nine days after infection, the culture treated with the C28 oligomer began to show evidence of toxicity. Therefore, we split the same number of cells and treated them with 1 μM, 0.5 μM, and 0.1 μM C28 oligomer. Fig. 4 demonstrates that after one passage, <5% of the cells were viable under treatment with 1 μM and 0.5 μM C28; however, cells treated with 0.1 μM C28 recovered. After the second passage, there were no viable cells in the cultures treated with 1 μM and 0.5 μM C28, whereas the 0.1 μM-treated cells remained healthy. The persistent antiviral effect of C28 oligomer, therefore, may not reflect a sequence-specific inhibition of HIV gene expression but may be due to a cytotoxic effect. It is worth noting, nevertheless, that the primer for the second strand synthesized by the RT is a polypurine tract sequence.

Selection of an Effective Target. The *in vitro* test provides a system in which to study the antiviral effects of drugs and to determine efficacy before *in vivo* studies. Our results indicate that the best targets for antisense inhibition may be directed against mRNAs of overlapping regulatory genes. The target sequences must be highly conserved between different HIV isolates to minimize the chance for virus escape because the isolates differ, even within one patient. Targeting a noncoding sequence (like SA) allowed the virus to escape. Virus replication was, however, in this instance, lower compared with the controls. An interesting and effective target appears to be a functional RNA, such as RRE. Chin (23) has provided evidence that the RRE structure is disrupted by antisense oligonucleotide interaction, which can block formation of the Rev–RRE complex *in vitro*. Our long-term tissue culture results support this mechanism. With the RRE-treated cultures, virus replication was always about 5–10 times higher, as compared with the rev-1-treated cells. Because multimerization of the Rev protein is required for

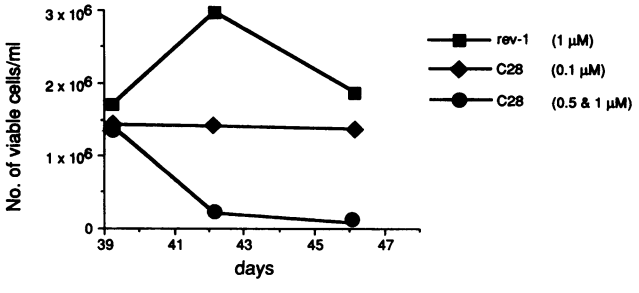

FIG. 4. Cytotoxic effect of the C28 oligomer.

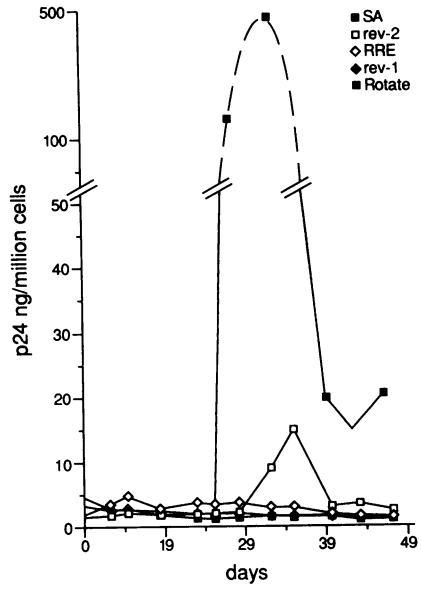

FIG. 5. Sequential treatment of HIV-1-infected cells with different antisense oligomers compared with repeated treatment with a single oligomer. Rotate indicates that at every treatment a different oligomer was added to the culture at 1 μM concentration. The sequence of treatment was RRE, rev-2, rev-1, and SA oligomers.

Rev function (24), a critical amount of Rev must escape from antisense inhibition for virus reproduction. However, if only a single RRE escapes from the antisense block, Rev might recognize RRE and activate virus expression. The oligomer directed against the *gag* gene could also inhibit HIV-1 replication efficiently, without generating what we call escape mutants. Sequences essential for packaging, situated around the *gag* initiation codon, have been shown to form a stable secondary structure (25). The gag oligomer may disrupt these structures, inhibiting viral packaging in addition to translation of gag mRNA.

Model for Treatment. HIV-1 has a high mutational rate and, therefore, all drugs designed to treat virus infection might induce the formation of escape mutants. To overcome this problem, combination chemotherapy has been suggested for treating HIV-1-infected patients. This therapy involves more than one drug directed against different targets, such as RT inhibitors combined with protease inhibitors. Antisense treatment of HIV-1-infected individuals, even when a highly conserved region is targeted, may result in the formation of escape mutants. Targeting different sequences, either in combination or in sequential treatment schedules, would place different selection pressures on the virus with little time to mutate an escape. The first treatments could consist of a mixture of oligomers or one targeted to a highly conserved region; the subsequent treatments could consist of a sequential administration of alternatively targeted oligomers. We have evaluated the beneficial effects of sequential treatment using antisense oligonucleotides directed against different sequences of the viral RNA. We have tested sequential treatment with RRE, rev-2, rev-1, and SA, designated as Rotate in Fig. 5, oligomers. During this study SA-treated virus escaped after 32 days, and rev-2-treated cells showed a higher level of virus replication compared with the rev-1- or RRE-treated cells. Sequential treatment kept virus replication at the level of the rev-1-treated cells, which when used alone gave the best antiviral activity among these oligomers. Our results suggest that the use of a combination of antisense oligonucleotides may be useful for treating HIV-infected patients.

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