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Inhibition of Human Immunodeficiency Virus Type 1 Rev-Rev-Response Element Complex Formation by Complementary Oligonucleotides

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Complementary 18-mer oligodeoxynucleotides (oligonucleotides) specifically inhibited the formation of human immunodeficiency virus Rev-Rev-response element (RRE) complexes. Inhibition of Rev-RRE binding required blockage of G-7819 to G-7820 in band shift assays. Structural studies revealed both local and distal effects. RRE structure was also disrupted by oligonucleotides targeted to other minor stems, by altering RNA renaturation conditions, or by reducing Rev concentrations—indicating a dynamic RRE structure and involvement of a minor RRE stem in the maturation of initial Rev-RRE complexes. Thus, complementary oligonucleotides alter RRE structure and may prove useful for the design of therapeutic anti-RRE oligonucleotides.

Regulation by protein-RNA interactions in a number of eukaryotic systems has recently been described (9, 17). In the human immunodeficiency virus (HIV), binding of the virally encoded Rev protein to the Rev-response element (RRE) (7, 11, 18, 31, 34) regulates viral replication (9). Both Rev and the RRE are required for expression of structural proteins, Gag and Env (15, 28, 39), from unspliced and singly spliced viral RNAs (12, 28). Rev has been proposed to enable transport of incompletely spliced transcripts directly by facilitating their export (29) or indirectly by disrupting aberrant splicesomes bound to these transcripts (5, 25). Rev may also stabilize partially spliced nuclear transcripts (15, 41) and improve the translational efficiency of RRE-containing RNAs in the cytoplasm (1, 2).

While two to eight Rev molecules bind to the RRE (11, 18, 30), the process of maturation from initial complexes is unknown. Studies have shown that an arginine-rich region of Rev is responsible for RNA binding to either intact 240-nucleotide (nt) RRE (31, 45) or 88- to 90-nt RRE subregions (10, 18, 20). Other Rev domains participate in activation (2, 26) or multimerization (27, 35). Analyses of the RRE suggest that both specific RNA sequences (18, 19) and RRE structure are required for Rev binding (13, 30, 36, 41). Mutations within a 9-nt region of stem II-a destroy its ability to bind Rev or facilitate its function (19). We reasoned that anti-RRE oligonucleotides could define essential bases within this region and reveal RRE features that may serve as unique targets for therapeutic oligonucleotides. To avoid the problems of nuclease sensitivity, membrane permeability, and the nonspecific binding of oligonucleotides with proteins at the cell surface and/or cytoplasm (4), in vitro assays were used to define optimal anti-RRE oligonucleotides (obtained from Gilead Sciences, Foster City, Calif.). These studies identified a class of oligonucleotides that blocks Rev-RRE binding and another class that disrupts preformed, mature Rev-RRE complexes. We also show that structural changes in a nonessential stem of the RRE may be required for maturation of the initial Rev-RRE complex.

The oligonucleotides used in this study (shown in Table 1) (3′-end-capped with methoxyethylphosphoramidate [MEA] linkages; oligonucleotides a1 to a6 also had amino-modified 5′ termini) were initially characterized by band shift assays using purified HIV type 1 (HIV-1) Rev (a gift of Z. Hostomska and Z. Hostomsky, Agouron Pharmaceuticals) and radioactive, synthetic RRE RNA derived from the minimal 240-bp HIV SF-2 RRE. The RRE was subcloned by the polymerase chain reaction (33) method using primers that included unique flanking restriction sites (*Bgl*II and *Sal*I sites were placed 5′ to nt 7767 of the HIV SF-2 RRE while *Xho*I and *Kpn*I sites in pRBK36 were placed 3′ to nt 8013). The (+) RRE RNA strand was synthesized with T7 RNA polymerase (Stratagene) using *Asp* 718-linearized vector DNA, while the (−) RRE RNA strand was derived from *Xba*I-linearized vector with T3 RNA polymerase by using standard methods (22).

A standard band shift assay utilized denatured RRE RNA at 85°C for 3 min in water and then cooled to 37°C 30 s prior to adding an equal volume of buffer B (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.3], 280 mM KCl, 0.5 mg of bovine serum albumin per ml, 50 μg of tRNA per ml, 10 U of Inhibit-Ace [Five-Prime, Three-Prime] per ml). In some experiments, RNA was denatured in the presence of oligonucleotides or renatured to 20 or 4°C. Inclusion of 2 mM $MgCl_2$ in buffer B did not alter the results. The RNA was renatured (15 min, 37°C) prior to dispersal into 10-μl aliquots to which oligonucleotides or Rev were added (17 ng of Rev with 0.5 to 1 ng of ^{32}P-labeled RRE RNA, 15 min). Since the temperature of RNA renaturation affected oligonucleotide efficacy in band shift assays, 37°C was the primary experimental temperature for other assays.

Oligonucleotides can block Rev-RRE complex formation. To detect partial inhibition of RRE band shifting, we chose Rev concentrations that shifted >90% of the RRE to the uppermost, mature band (Fig. 1A). We and others (10, 11, 21) noted that low Rev concentrations resulted in partially retarded, intermediate RRE bands (Fig. 1A, lanes 3 and 4). As shown in Fig. 1B, a region IIa oligonucleotide blocked the Rev-induced RRE band shift in a concentration-dependent manner. Inhibition of the Rev-RRE band shift was sequence dependent (Fig. 1C and D). Ineffective oligonucleotides included those beyond nt 7820 (Fig. 1B, lanes 5 to 7, and Fig. 1C, lanes 14 and 15) and a missense oligonucleotide (Fig. 1C, lane 8, and Table 1).

From the pattern of inhibition by overlapping oligonucleotides, we conclude that nt 7821 to 7848 of the RRE

TABLE 1. Anti-RRE oligonucleotides

Oligonucleotide[a]	Sequence (5′ to 3′)[b]	Result[c]
Stem IIa/b		
	^{7811}agcactatgggcgcagtgtcattgacgctgacggtaca (+RNA strand)	
	tgtaccgtcagcgtcaatgacactgcgcccatagtgct^{7811} (−strand)	
a1	.....cactgcgcccatagtgct^{7811}	++
10	.....gacactgcgcccatagtg^{7813}	++
a2	.....atgacactgcgcccatag^{7815}	+
11	.....caatgacactgcgcccat^{7817}	+
a3, 12	.....gtcaatgacactgcgccc^{7819}	+
13	.....agcgtcaatgacactgcgc^{7821}	−
14	.....cagcgtcaatgacactgc^{7823}	−
a4	.....tcagcgtcaatgacactg^{7824}	−
a5	...accgtcagcgtcaatgac^{7828}	−
a6	tgtaccgtcagcgtcaa^{7832}	−
M	.....ctacgaccgccgtcgatt	−
Non-stem IIa/b (stem)		
79c (I)	tcctgctgctcccaagaa^{7793}	P+
79d (IIc)	gacaataattgtctggcc^{7849}	−
79e (IIc-III)	tgctgttgcactatacca^{7867}	−
78h (III)	caaattgttctgctgttg^{7877}	−
79a (IV)	cagatgttgttgcgcctc^{7907}	−
79b (V)	cttgatgccccagactgt^{7934}	P
90g (V)	actcttgcctggagctgc^{7954}	P++
78f (I)	aggtatctttccacagcc^{7972}	P
78g (I)	agggagctgttgatccct^{7991}	−

[a] Oligonucleotide M is a missense oligonucleotide with the same nucleotide content as a1 but in a randomized array.

[b] The minimal 38-nt RRE stem IIa/b (18) is shown 5′ to 3′ from nt 7811 of the HIV SF2 sequence. Numbers adjacent to oligonucleotide sequences indicate the complementary RRE base positions.

[c] Oligonucleotide efficacy was evaluated in Rev-RRE band shift assays. Inhibition of the band shift was determined by densitometry. ++, strong inhibition (50% block of Rev-RRE complexes at 1 to 2 μM oligonucleotide); +, moderate inhibition (50% block at 8 to 10 μM). Partial inhibition of band shifting (P) resulted in smearing or broad intermediate bands. P+, band shift migrating just below the maximal Rev-RRE band shift; P++, band shifting >2 band widths below the uppermost band.

sequence spanned by stem IIa/b oligonucleotides are dispensible (common to oligonucleotides a4, a5, a6, 13, and 14) while nt 7811 to 7820 (AGCACUAUGG) are required for blocking Rev binding (common to a1, a2, and 10 to 12). While bases complementary to nt 7811 to 7818 (shared by a1, a2, 10, and 11) improved the inhibition of Rev-RRE band shifting, oligonucleotide 12 lacks these bases but still blocked the Rev-RRE band-shift. Since oligonucleotide 13 was ineffective and lacks the two terminal cytosine residues of oligonucleotide 2, the efficacy of oligonucleotide 12 resides in one or both of these bases; hence we conclude that G-7819 to G-7820 of the RRE are crucial for initial Rev binding. These bases are within the sequence CACUAUGGG (nt 7813 to 7821), previously described as essential for RRE activity in vivo (19).

The simplest explanation for these results is that sequence-specific DNA binding to the RRE prevents subsequent Rev binding. Alternatively, Rev-DNA complexes are possible at high DNA concentrations (11), but three lines of evidence suggest that the former mechanism predominates: ^{32}P-oligonucleotide-RRE complexes form (not shown), RNase H specifically cleaves oligonucleotide-^{32}P-RRE complexes (not shown), and inhibition of complex formation by the blocking oligonucleotides required a precise order of addition (discussed below). Additionally, oligonucleotide 90g altered Rev-RRE complex migration even when added to preformed complexes (Fig. 2A, lanes 8 and 9), consistent with predominant DNA-RNA interactions. Unlike stem IIa/b oligonucleotides with respect to blocking effects, oligonucleotide 90g represents a second class of compounds that do not block Rev-RRE binding but alter, without

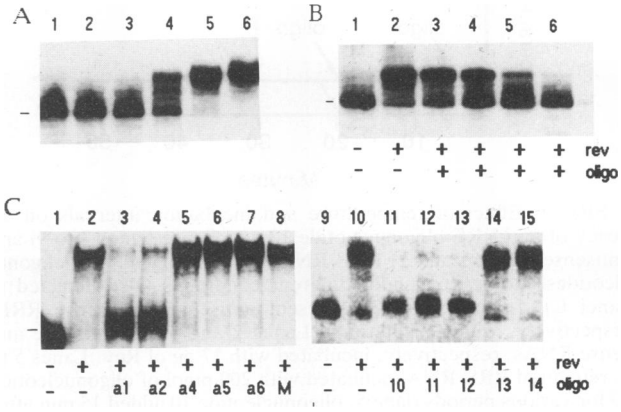

FIG. 1. Band shift assay of HIV type 1 Rev and RRE. (A) Rev-RRE band shift is concentration dependent. One nanogram (12 fmol) of sense ^{32}P-RRE RNA was incubated with purified Rev as described in Materials and Methods, in the following amounts (nanograms): lanes 1, none; lane 2, 0.67; lane 3, 2; lane 4, 6; lane 5, 17; lane 6, 51. (B) Inhibition of Rev-RRE band shift by region IIa oligonucleotide 10. RRE RNA renatured at 37°C for 15 min was incubated with oligonucleotide 10 at 0, 3, 10, 30, and 100 pmol (lanes 2 to 6, respectively) for 15 min. Rev (17 ng, 0.8 pmol) was added, and the mixture was incubated for 15 min at 37°C prior to electrophoresis (11). (C) Sequence-dependent inhibition of Rev-RRE band shift by region IIa/b oligonucleotides. Two hundred picomoles of the oligonucleotides described in Table 1 were sequentially incubated with RRE RNA and Rev as described in the legend to panel B. In all panels, the line indicates the position of free RRE RNA.

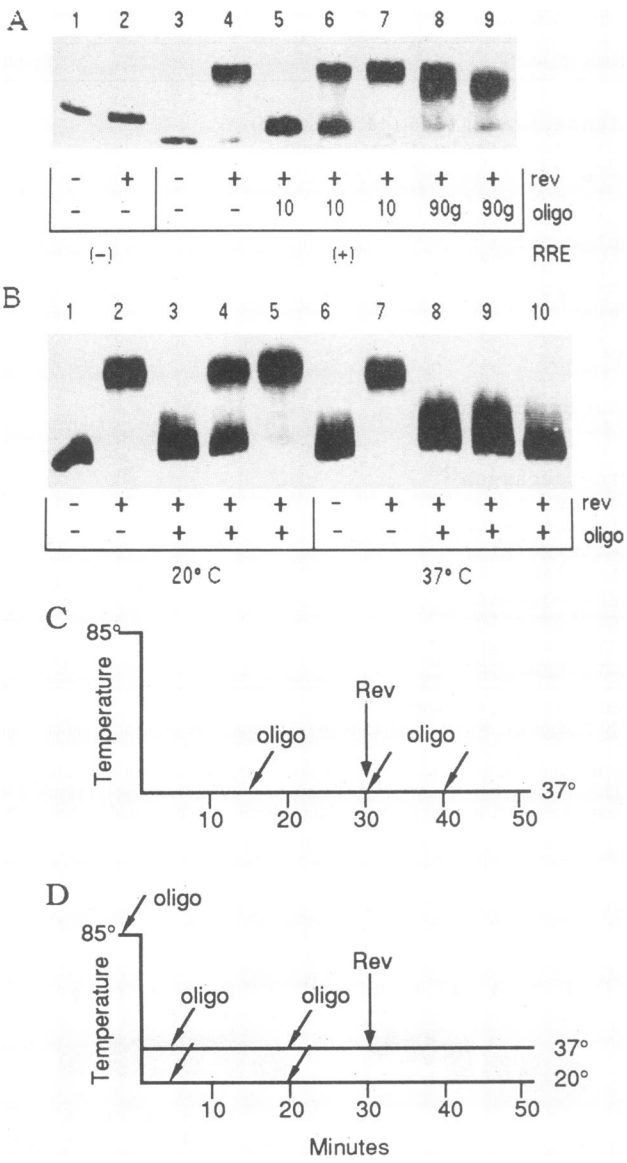

FIG. 2. Effect of temperature and incubation intervals on efficacy of anti-RRE oligonucleotide 10. (A) Sense (lanes 3 to 9) and antisense (lanes 1 and 2) RRE RNA were incubated with oligonucleotides and/or Rev under different conditions (also depicted in panel C). Lanes 1 and 3 represent antisense and sense RRE, respectively, with no additions. Lanes 2 and 4 are antisense and sense RNAs, respectively, incubated with 17 ng of Rev. Lanes 5 to 7, renatured RRE RNA incubated with 200 pmol of oligonucleotide 10 for various periods (lane 5, oligonucleotide 10 added 15 min after renaturation; lane 6, oligonucleotide 10 and Rev added simultaneously to the RRE after 30 min of renaturation at 37°C; lane 7, oligonucleotide 10 added 10 min after Rev was added to the RNA). Lanes 8 and 9 show RRE RNA incubated with oligonucleotide 90g 15 min prior to Rev (lane 8) or 10 min after (lane 9) Rev addition. (B) Effect of renaturation temperature upon oligonucleotide 10 inhibition of Rev-RRE band shift. As depicted in Fig. 2D, RRE RNA was denatured at 85°C and renatured at either 20°C (lanes 1 to 5) or 37°C (lanes 6 to 10) and incubated with Rev (lanes 2 to 5 and 7 to 10) and oligonucleotide 10 under the following conditions: to lanes 3 and 8, 200 pmol of oligonucleotide 10 was added to the RRE prior to denaturation; to lanes 4 and 9, oligonucleotide 10 was added 5 min after renaturation at indicated temperatures; to lanes 5 and 10, oligonucleotide was added 20 min after renaturation. Rev (17 ng) was added to the lanes 30 min after the temperature was shifted down from 85°C. Note that oligonucleotide 10 is less effective with RRE renatured at 20°C (lanes 4 and 5) than with RRE at 37°C (lanes 9 and 10).

complete disruption, preformed complexes. This second class of oligonucleotides causes one or more central intermediate RNA bands within a smeared region (Fig. 2A and Table 1).

Order of addition and temperature of renaturation affects inhibition. It is unknown if the steady-state RRE structure is unique or if it alternates between metastable structures in vivo before or after Rev binding; multiple stable (~100 ± 10 kcal [ca. 418 kJ]/mol) RRE structures can be derived from energy minimization predictions (47). Such heterogeneity might be accentuated by bound oligonucleotides and result in band smearing or intermediate band shifts. Hence, studies were performed under a variety of renaturation conditions to reduce structural heterogeneity. No effect of magnesium ions was found, but the conditions of renaturation and time of Rev addition affected the inhibition of Rev-RRE complexes by stem IIa/b oligonucleotides. In contrast to the strong inhibition found with preincubations (Fig. 1B and C and Fig. 2A), simultaneous or post-additions severely reduced or abolished inhibition (Fig. 2A, lanes 6 and 7). This order effect was sequence dependent since oligonucleotide 90 g (specific for stem V) was independent of the time of Rev addition (Fig. 2A, lanes 8 and 9).

The temperature of RNA renaturation and duration of preincubation controlled oligonucleotide efficacy. Inhibition of the Rev-RRE band shift by oligonucleotide 10 was strongest when RRE was renatured at 37°C (e.g., compare lanes 5 and 10 of Fig. 2B). Additionally, inhibition was dependent upon the preincubation time (compare lanes 4 and 5 with lanes 9 and 10 of Fig. 2B). If oligonucleotide was included during denaturation, inhibition occurred at both renaturation temperatures (compare lanes 3 and 8 of Fig. 2B).

Oligonucleotides alter RRE structure. Both T_1 and cobra venom ribonucleases were used to characterize the effects of oligonucleotides and Rev upon RRE structure, but RNase CV results are shown since they were definitive for oligonucleotides with blocking activity. Briefly, 120 to 160 ng of 5′-^{32}P-labeled RRE RNA was incubated (15 min, 37°C) with oligonucleotides (20 μM) prior to the addition of Rev (50 ng in 10 μl, 37°C, 15 min). Samples were digested (14 min, 37°C) in buffer B supplemented with 0.1 M NaCl–1 mM $MgCl_2$ with RNase CV (0.002 U/ml; Pharmacia), deproteinized, and precipitated prior to electrophoresis in 6% polyacrylamide–7 M urea gels run with a buffer gradient (3). A RNA sequencing ladder was generated with end-labeled RNA and an enzyme-based RNA sequencing kit according to the manufacturer's instructions (Pharmacia). Readings from 5′-labeled RRE RNA were reliable only to nt G-7926.

Treatment of Rev-RRE complexes with a ribonuclease specific for double-stranded RNA (dsRNA), RNase CV, protected a domain from nt 7796 to nt 7814 (regions 5 to 7 in lanes 1 and 2 of Fig. 3). Within this domain were several bases inaccessible to RNase CV (G-7809, C-7813, and A-7813) and one RNase T_1-sensitive site (G-7802) that was invariant with Rev addition; structural results are summarized in Fig. 4 and Table 2. Encompassing this domain were several other strong RNase CV-reactive bands extending from nt 7790 to 7881. Some RNase CV-reactive bands were diminished by Rev (Fig. 3A, lane 2; Fig. 3B, lanes 4, 6, and 10; Table 2). Since RNase CV is sequence independent,

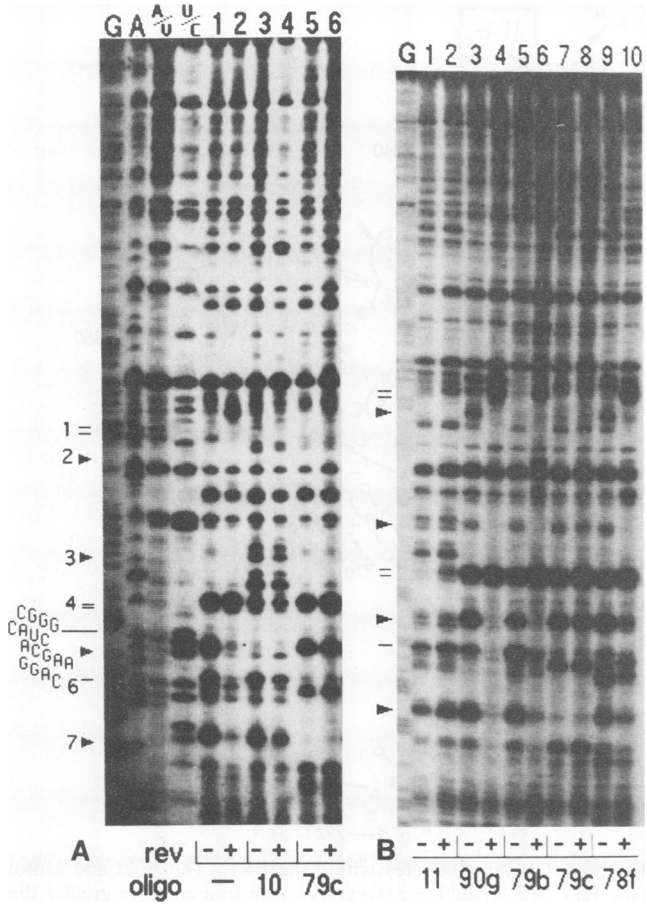

FIG. 3. RNase CV sensitivity of RRE in the presence of anti-RRE oligonucleotides. (A) Lanes 3 to 8, oligonucleotides incubated with renatured RRE RNA (15 min, 37°C) prior to the addition of Rev (50 ng, 15 min, even-numbered lanes) and digested with RNase CV (20 min, 37°C). Lanes 3 and 4, oligonucleotide 10; lanes 5 and 6, oligonucleotide 79c. (B) RRE RNA was renatured in the presence of 1 mM $MgCl_2$. Lanes 1 and 2, oligonucleotide 11; lanes 3 and 4, oligonucleotide 90g; lanes 5 and 6, oligonucleotide 79b; lanes 7 and 8, oligonucleotide 79c; lanes 9 and 10, oligonucleotide 78f. Symbols at left of the G channel indicate regions of Rev-dependent changes in the RNase CV reactivity (Table 2). Symbols, from top to bottom, are the same as in panel A. A portion of the stem IIa/b sequence is shown. A dot indicates C-7815 between lanes 2 and 3 in panels A and B.

bands hidden by Rev are either Rev contacts or nuclease inaccessible because of RNA structure. Conversely, other bands appeared or intensified after Rev binding; e.g., region 1 (C-7877, G-7876) in 3′ stem IIa, C-7856 within stem IIc, and A-7825, U-7827 within stem IIb. These sites may represent dsRNA regions with improved RNase CV access. Our results are consistent with RRE predictions (23, 29) and structural studies of HIV HXB3 RRE (21), but differences in deduced structure result from methodology (such as temperature; see below) or strain variation (in particular, see A-7825 and U-7827 in Fig. 4).

Most Rev-induced changes in RRE structure were eliminated by anti-RRE oligonucleotides. In addition, RRE-oligonucleotide complexes also revealed changes in the target sequences and flanking regions in the absence of Rev; oligonucleotide 10 significantly reduced the intensity of RNase CV-reactive bands within the target 18-mer sequence (C-7813-C-7815 in region 5; Fig. 3A, lanes 3 and 4) to the intensity of CV-reactive bands found with RRE and Rev (Fig. 3A, lane 2). Many new, Rev-independent RNase CV bands also appeared outside the target site (Fig. 3A, lanes 3 and 4). Rev-sensitive bands (regions 2, 3, 5, 6, and 7 in Fig. 3) remained constant in the presence of stem IIa oligonucleotides (Fig. 3A, lanes 3 and 4). Bands that appeared or intensified (regions 1 and 4) after Rev addition remained constant in the presence of IIa oligonucleotides.

Stem III changes caused by temperature or oligonucleotide 90g. While 90g had no effect upon the Rev-dependent RNase CV sites in regions 1 to 7, strong bands appeared at nt 7880 and 7883 above region 1 within stem III (Fig. 3B, lanes 3 and 4). The disruption of stem III by 90g was sequence specific since 79b, specific for the opposing strand, did not induce stem III reactivity (compare Fig. 3A, lane 1, with Fig. 3B, lanes 3 and 5). An analysis of RRE RNA renatured at reduced temperatures, described below, revealed that RNA renaturation at 4°C caused similar changes.

RRE RNA was renatured at various temperatures to help analyze oligonucleotide-induced changes in RRE structure. Several qualitative and quantitative changes in protected bands within dsRNA regions were found (Fig. 5). New bands or bands of increased intensity were found when the RRE was renatured at 20 or 4°C and assayed for RNase CV reactivity in the absence of Rev; the major differences between lanes 1, 3, and 6 of Fig. 5 are summarized in Table 2. At 4°C, changes were found at multiple sites: RRE stem I (nt 7780), between stem I and IIa (nt 7804 to 7815), and, in particular, stem III at nt 7880 and 7883 to 7885 above the region 1 protection site (Fig. 4 and Fig. 5, lane 4) which are similar to stem III changes caused by 90g. Since oligonucleotide 90g caused intermediate band shifts with any order of addition (Fig. 2), we conclude that changes in stem III reflect structural RRE alterations essential for the maturation of initial Rev-RRE complexes to the slowest band shift.

Clarifying Rev binding to specific RRE regions is important to understanding both the mechanism(s) of viral RNA export and potential pharmacological agents. Our results support and extend previous work establishing stem IIa and the flanking G-7819 to G-7820 region as important parts of the Rev recognition site (19). In addition, our results are consistent with the originally proposed RRE structure (29). However, our model differs slightly from this and a recently modified model suggesting a larger central loop (21). In this report, the conserved C-7902 of HIV SF2 was RNase CV reactive, which indicates a short stem, C-7902 to U-7903:A-7966 to G-7967 may form in this region. Additionally, the RNase CV reactivity of C-7847 suggests that an internal loop within stem IIb is flanked by the double-stranded U-7818 to G-7819:C-7847 to A-7848 region.

Unlike a previous structural analysis of the HIV HXB3 RRE (21), we found in HIV SF2 RRE (i) decreased strong stem I RNase CV reactivity with Rev addition, (ii) increased RNase CV reactivity in the 3′ side of stem IIa with Rev addition, and (iii) increased reactivity in stem IIa/b and the neighboring stem III region by altering the conditions of RRE renaturation. All of these changes were blocked by stem IIa oligonucleotides that targeted G-7819 to G-7820. The extensive Rev-dependent changes in stem I are consistent with studies showing its secondary role in mediating Rev function (13, 31, 36). These results can be explained by secondary Rev binding sites that follow Rev multimerization upon the RNA (27).

We have shown that RRE structure and oligonucleotide-

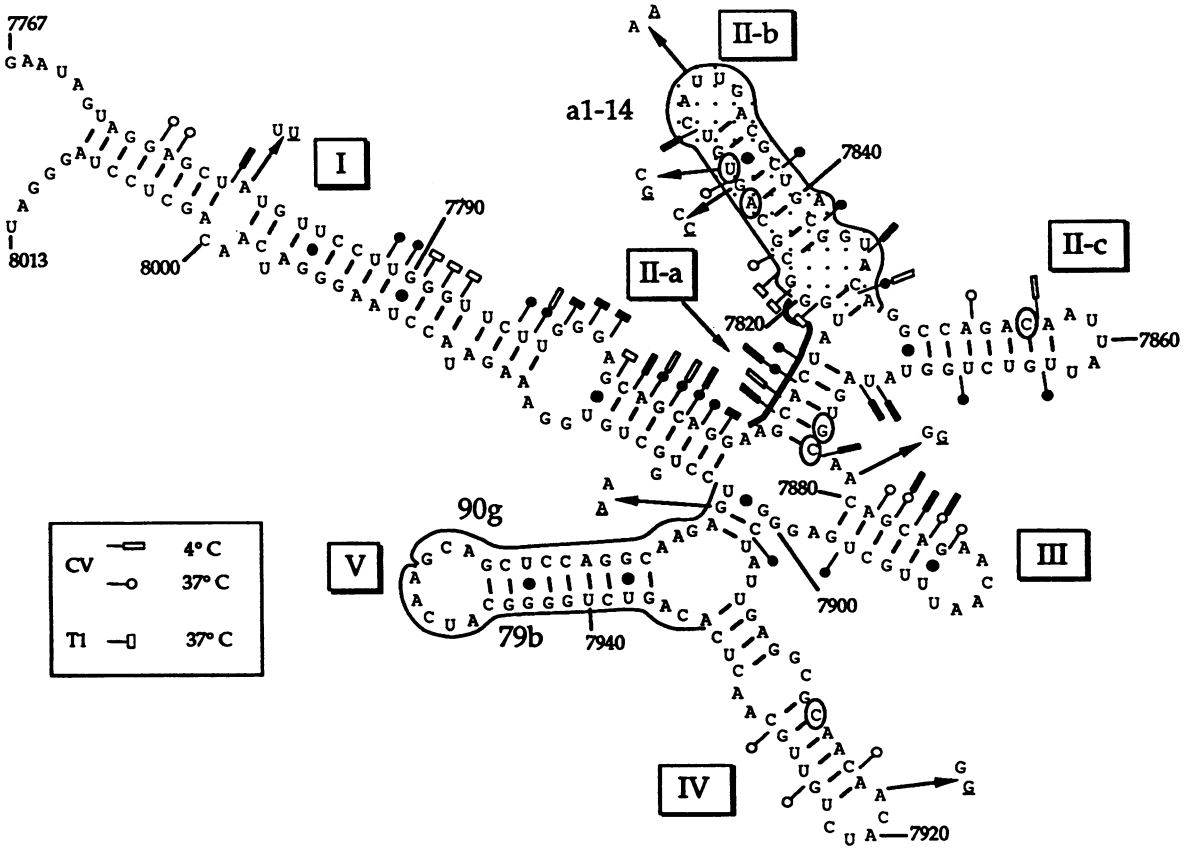

FIG. 4. Diagram of (A) RRE and anti-RRE oligonucleotides and (B) RNase CV-reactive sites within the RRE. The Fold and Mufold RNA-structure predicting programs of Zuker (47) were used to analyze the HIV SF2 RRE. Four structures with free energies greater than −90 kcal (ca. −376 kJ)/mol were found, and the depicted structure was modified according to RNase CV results. The predicted stems are indicated (I, IIa, IIb, IIc, III, IV, V). Introducing the results of nuclease protection studies into the most stable predicted structure reduced the predicted free energy to −88 kcal (ca. −368 kJ)/mol from −93 kcal (ca. −389 kJ)/mol for HIV SF2. Variations in HIV-1 sequence are shown by arrows; alternate bases for HIV HXB3 and HIV BRU (underlined) are indicated. The positions of selected oligonucleotides are shown by flanking lines (90g, 79b, and all stem IIa/b oligonucleotides). The dot pattern over stem IIa/b describes the region in Table 1. The thick line from nt 7811 to 7820 corresponds to the common region targeted by oligonucleotides a1, 10, a2, 11, and 12 that blocked the Rev-RRE band shift. The RNase CV (at 37 and 4°C) and RNase T_1 reactive sites of the RRE in the presence of Rev are shown from six RNase CV and four T_1 experiments. Filled symbols indicate sites whose intensity diminished in the presence of Rev. Circled nucleotides indicate sites that appeared or showed intensified RNase CV reactivity upon Rev binding.

RRE band shift behavior is strongly influenced by sequence, temperature, and order of addition. These results suggest the RRE structure may be both dynamic and composed of complex tertiary interactions like other complex RNA structures that show temperature-dependent structural changes (42). The deduced tertiary RRE interactions could result from stem-stem interactions; e.g., RRE stems V and III may have direct contact via five of six bases (nt 7891 to 7897, UUUGCUG, and nt 7945 to 7951, CAUCAAG) where the sequence is conserved.

The role of stem III or other minor RRE stems enabling Rev function is unknown. A twofold loss of Rev function results from either precise excision of stem III (13) or removal of stem III within the context of stem IV and V deletions (41). Stems IV and V account for only a twofold effect upon Rev activity (41) but they may compose the recognition site for another RNA binding protein, the Rex protein of human T-cell leukemia virus type I (41), which can complement Rev-deficient HIV strains (16, 38). Thus, while Rev and Rex are specific for different regions of the RRE, stem III contributes a twofold effect upon both Rev or Rex (41). Consistent with a conserved role enabling both Rev and Rex activity, structural changes in stem III induced by oligonucleotide 90g (specific for stem V) could reveal additional low-affinity Rev sites (21) or enable local Rev multimerization (27). From either model, such tertiary interactions in Rev and the RRE may affect the kinetics or stability of Rev multimerization and RNA export.

Emergence from a latent phase of HIV infection may require a critical amount of Rev to trigger the export of incompletely spliced, nuclear viral transcripts (37). RNA transport may only occur with RRE-containing RNAs fully decorated with Rev multimers (27). Levels of Rev in the nucleus can be modulated by regulating its synthesis, localization, or bioavailability within the nucleus. A Rev-binding, nucleolar protein may help transport Rev into the nucleus (14) or regulate free, nuclear Rev levels. When Rev is still limiting, oligonucleotides that block G-7819 to G-7820, as shown in this study, should prevent the continued formation of new Rev-RRE complexes. Upon elevated Rev synthesis, agents that disrupt stem III (such as oligonucleotide 90g)

TABLE 2. Effects on RRE reactivity to RNase CV

Region[a]	Result[b] with oligonucleotide[c] (renaturation temp)[d]						
	None (4°C)	None (37°C)	a6 (37°C)	BL[e] (37°C)	90g (37°C)	79b (37°C)	79c (37°C)
1	±/++	−/++	+/+	+/+	±/++	−/++	−/−
2	+/−	+/−	−/−	−/−	+/−	+/−	±/±
3	±/±	+/−	−/−	−/−	+/−	+/−	+/+
4	+/+	−/+	±/±	±/±	−/+	±/±	+/+
5	+/±	++/−	±/±	±/±	++/−	++/−	++/++
6	+/±	++/−	±/±	±/±	++/−	++/−	++/++
7	±/±	++/−	++/++	++/++	++/−	++/−	++/++

[a] Symbols in Fig. 3 and 5, described numerically below, correspond to RNase CV-reactive bands in RRE renatured at 37°C: 1 (=), nt 7876–7877, bands appear with Rev; 2 (▶), nt 7863 and 7867, bands diminish; 3 (▶), nt 7838, band diminishes; 4 (=), nt 7825 and 7827, bands intensify or appear; 5 (▶), nt 7814 and 7815, bands diminish; 6 (−), nt 7804–7807, bands diminish; 7 (▶), nt 7795–7796, bands diminish.

[b] Result with Rev/result without Rev. ++, very strong band; +, strong band; ±, weak band; −, no band.

[c] Summary of data from Fig. 3A and B and six other experiments.

[d] Summary of data from Fig. 5 and three other experiments.

[e] BL; oligonucleotides from the RRE stem IIa/b region described in Table 1 that block Rev-RRE complex formation, including a1, a2, 10, 11, and 12.

could block the maturation of Rev-RRE complexes and RNA transport.

The prospects for therapeutic application of these anti-RRE sequences are daunting in view of serum nucleases (40) and the problem of membrane permeability. Advances in internucleoside linkages have led to several exonuclease-resistant oligonucleotides which include phosphorothioates, phosphoramidates, and methylphosphonates (46). However, entry of current oligonucleotides into the cytoplasm in significant levels from the medium remains a major hurdle (8). Recent studies have shown that cytoplasmic injection of oligonucleotides leads to their quantitative, nuclear accumulation by a diffusion-limited process; hence, nuclear oligonucleotide concentrations at levels used in our assays are likely attainable (6) once the membrane permeability problem is solved. Previous proposals for therapeutic oligonucleotides, including anti-Rev oligonucleotides, have focused upon antisense mechanisms and have designed oligonucleotides specific for translational initiation sites (32) based upon in vitro studies showing RNase H cleavage of the RNA-oligonucleotide hybrid (43). Other studies have reported activity of oligonucleotides specific for viral splice sites (44), but the overall efficacy and specificity of oligonucleotides delivered by addition to the medium is complex. With the nuclear retention of oligonucleotides (6, 24), the exact mechanism of antisense or antisplicing oligonucleotides delivered to endosomes or lysosomes remains uncertain. Until improved delivery methods or membrane-permeant oligonucleotide linkages are devised, the specificity of antiviral oligonucleotides may best be determined using in vitro assays.

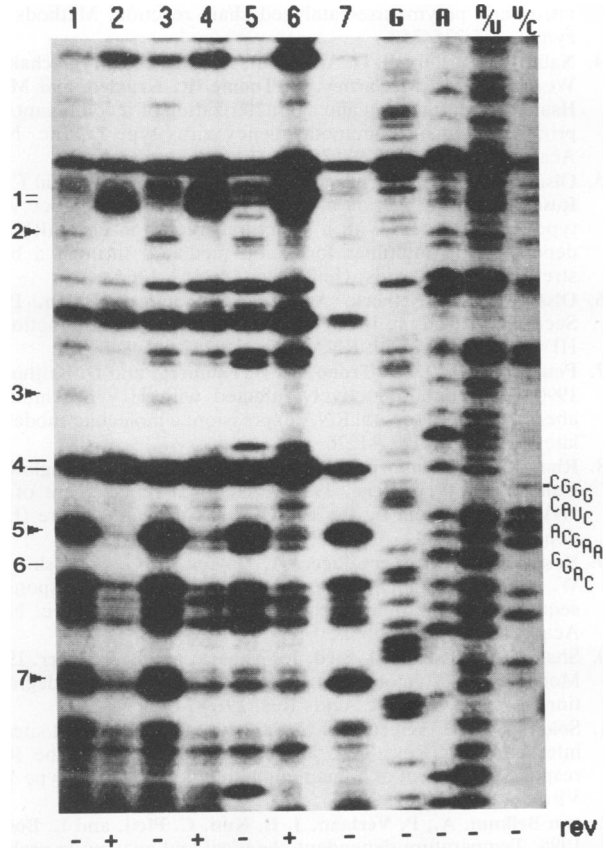

FIG. 5. Temperature-dependent RNase CV reactivity of sense RRE RNA. End-labeled RRE (120 ng) was renatured at 37°C (lanes 1, 2, and 7), 20°C (lanes 3 and 4), or 4°C (lanes 5 and 6) for 15 min prior to incubation with 51 ng of Rev (even-numbered lanes) for 15 min at indicated temperatures. RNase CV was added to all samples and digested for 20 min at 37°C.

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