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Minimal Rev-Response Element for Type 1 Human Immunodeficiency Virus

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Rev protein regulates nuclear export of viral mRNAs that contain a 240-base RNA sequence termed the Rev-response element (RRE). We demonstrate that an 88-base truncated RRE encompassing a known Rev binding site can mediate Rev responsiveness in vivo. Two tandem copies of this mutant function as efficiently as the full-length RRE.

The Rev transactivator of type 1 human immunodeficiency virus (HIV-1) acts by inducing cytoplasmic expression of the incompletely spliced mRNAs that code for HIV-1 structural proteins (reviewed in reference 2). This posttranscriptional transactivation requires direct binding of Rev protein to a *cis*-acting locus in these mRNAs which is termed the Rev-response element (RRE) (11, 14). On the basis of functional mapping, Malim et al. (11) postulated that the RRE coincides with a 240-base region of RNA secondary structure in the *env* coding region; subsequent studies have confirmed both the existence of such a structure in vivo (4, 10) and its importance for transactivation (1, 3, 5, 6, 12, 13, 16). Rev protein binds specifically to a discrete 30- to 71-base region within the RRE (5, 12), and mutations that disrupt this binding region abolish transactivation (6, 12, 13). The importance of sequences elsewhere in the RRE, however, remains uncertain: mutations at a variety of sites in the 240-base element can severely inhibit Rev binding and transactivation (1, 3–6, 12, 13, 16), but it is not known whether these sites have independent functions or merely affect folding or accessibility of the Rev binding locus. To address this issue, we attempted to define the minimal sequence required for RRE activity. We now report that an 88-base truncated RRE encompassing the Rev binding site is sufficient to mediate transactivation in vivo and that two tandem copies of this mutant function as efficiently as the intact RRE. These findings imply that most of the RRE has no unique role in transactivation and suggest that Rev binding might be the only requirement for RRE function.

We have previously described (7, 8) a transient transfection assay for Rev activity based on an HIV-1-derived reporter plasmid (pDM128) in which the chloramphenicol acetyltransferase (CAT) coding sequence has been inserted into the 2.3-kb intron that contains the RRE. Unspliced transcripts of pDM128 harboring the CAT sequence are permitted to enter the cytoplasm only when Rev is present; thus, CV1 cells express only traces of CAT activity when transfected with pDM128 alone but yield approximately 100-fold-higher levels of CAT when cotransfected with a second plasmid (pRSV-Rev) coding for Rev (8). By deleting from pDM128 a 1.2-kb intron sequence that included the RRE, we derived a defective reporter (pDM138) that did not respond to Rev. We then compared the abilities of various RRE derivatives to restore transactivation when inserted at the site of this deletion in pDM138. As indicated in Table 1, the insertion of a 318-bp DNA fragment encompassing the entire 240-base RRE conferred a 23-fold mean CAT induction in response to Rev. The failure of this insertion to restore the full 100-fold response seen with pDM128 may suggest the presence of additional positive or negative regulatory sequences elsewhere in the deleted region. For the present study, however, a 23-fold CAT induction was considered the wild-type response attributable to the RRE.

The 240-base RRE sequence has been proposed (11) to form a double-stranded stem (stem 1) crowned by four stem-loops (SL2 to SL5), one of which gives rise to two subsidiary stem-loops designated SL2A and SL2B (Fig. 1B). Our findings, consistent with earlier reports, showed that mutants of the 240-base RRE lacking any of four stem-loops (SL2B, SL3, SL4, or SL5) could support transactivation but that precise deletion of SL2 or of SL2A alone reduced transactivation to very low levels (Table 1). These data confirm earlier reports that SL2A is the region most critical for RRE function. Like previous investigators (4), we observed that some RRE mutations also affected basal CAT activity in the absence of Rev, for reasons that are not known.

Although point mutations in and around SL2A can inhibit RRE function, compensatory mutations designed to preserve the folding pattern (Fig. 2, structure A) predicted by Malim et al. (11) have been found to restore both Rev binding and transactivation (12, 13). This suggests that the conformation of this region is a major determinant of Rev binding, although primary sequence is also known to play a role (6). A comparison of predicted RRE conformations among different HIV-1 strains, however, recently led Heaphy et al. (5) to propose an alternative structure for this region (Fig. 2, structure B). To test the validity of this alternative model, we constructed two additional mutants of the 240-base RRE. The first (designated het1) contained four consecutive point mutations that would completely disrupt structure B but would only partially destabilize structure A. This mutant was found to retain substantial RRE activity (Fig. 2). The second mutant (het1/het2) contained the het1 mutations along with four additional mutations that were predicted either to restore the base pairing of structure B or to further disrupt structure A. As depicted in Fig. 2, the het1/het2 mutant was completely unresponsive to Rev. This

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TABLE 1. Functional characterization of RRE mutants in a cotransfection assay

Construct[a]	Presence of Rev	Relative CAT activity[b]				Mean Rev response (fold)
		Expt 1	Expt 2	Expt 3	Expt 4	
pDM128	+			43.5, 53.0		110
	−			0.5, 0.4		
pDM138	+			6.2, 3.0		1
	−			4.4, 3.0		
240-base wild-type RRE	+	87.7[c]	94.6[c]	69.0, 47.6	72.1	23
	−	3.5	6.0	3.1, 1.9	3.2	
ΔSL2 (43–106)	+	22.6	69.0			2
	−	11.5	37.0			
ΔSL2A (53–72)	+	4.4	12.8			2
	−	2.1	7.3			
ΔSL2B (82–98)	+	28.4	94.7			8
	−	3.5	11.2			
ΔSL3 (111–126)	+	56.4	77.3			186
	−	0.5	0.3			
ΔSL4 (137–163)	+	6.2	4.0			9
	−	0.4	1.0			
ΔSL5 (168–191)	+	13.6	54.5			22
	−	0.6	2.5			
bull RRE						
1 copy	+			16.5, 10.2	29.8	6
	−			2.9, 2.2	4.5	
2 copies	+			47.7, 26.0	25.2[d]	22
	−			1.4, 1.4	1.8[d]	
4 copies	+			23.4, 17.7	21.7	7
	−			2.4, 2.8	5.9	
ΔSL2A (53–72)	+			5.2	1.9	<1
	−			5.5	5.1	
ΔSL2B (82 to 98)	+			5.1	2.6	<1
	−			4.2	5.6	

[a] Numbers in parentheses are the nucleotide positions deleted.
[b] Normalized in each experiment relative to an internal control plasmid as described previously (7, 8).
[c] Values exceed linear range of the assay and were not used in calculating mean response.
[d] Samples diluted fourfold to maintain linearity of the assay.

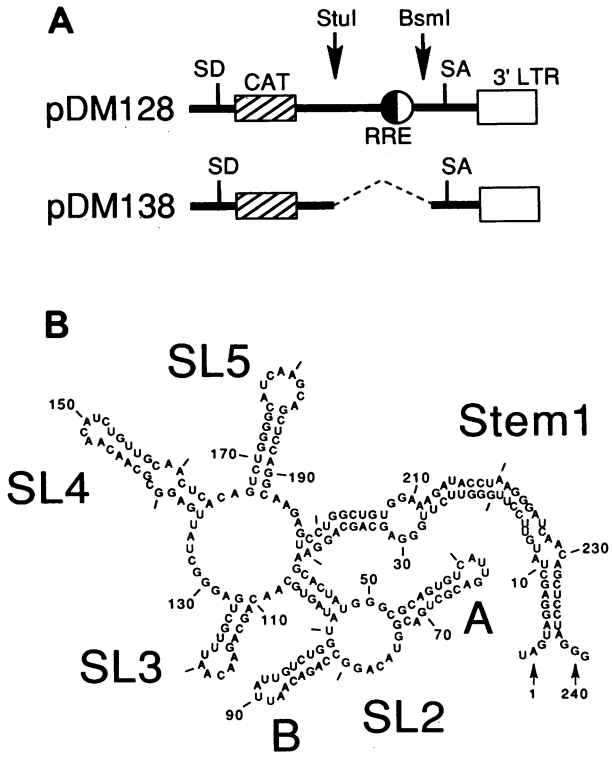

FIG. 1. Reporter constructs and the RRE. (A) The reporter pDM128 transcribes sequences from the 3′ half of HIV-1 SF2 (15) under control of the simian virus 40 promoter and enhancer; a 1.2.-kb *Stu*I-*Bsm*I fragment (HIV-1 nucleotides 6852 to 8066) was excised from pDM128 and replaced by a *Cla*I linker to produce pDM138. The unique splice donor (SD) and acceptor (SA) sites and the RRE are indicated. LTR. Long terminal repeat. (B) Predicted secondary structure (11) of the RRE from HIV-1 SF2. Residues 1 and 240 correspond to bases 7770 and 8009, respectively, of the viral genome. A 318-bp DNA fragment encompassing the RRE was prepared from pDM128 by the polymerase chain reaction with a pair of oligonucleotide primers with the sequences 5′-GGGGGATCCATCGATAGGCAAAGAGAAGAG-3′ and 5′-GGGGGATCCATCGATTGCAAATGAGTTTTT-3′; the amplified fragment corresponded to HIV-1 nucleotides 7725 to 8043, flanked on either side by a 15-bp linker containing *Cla*1 and *Bam*HI sites (underlined above). This fragment was trimmed with *Bam*HI and cloned into pUC11S for oligonucleotide-directed mutagenesis (9); wild-type or mutant RRE derivatives were then excised with *Cla*I and subcloned into the unique *Cla*I site of pDM138.

limited analysis thus favored structure A for the critical SL2A subdomain.

Having confirmed that SL2 is indispensable in the context of the 240-bp RRE, we examined whether this element might be sufficient for RRE function. We constructed an 88-base truncated RRE (designated bull) in which the wild-type SL2 sequence was fused to an 8-bp sequence from the base of stem 1, with all intervening and flanking residues deleted.

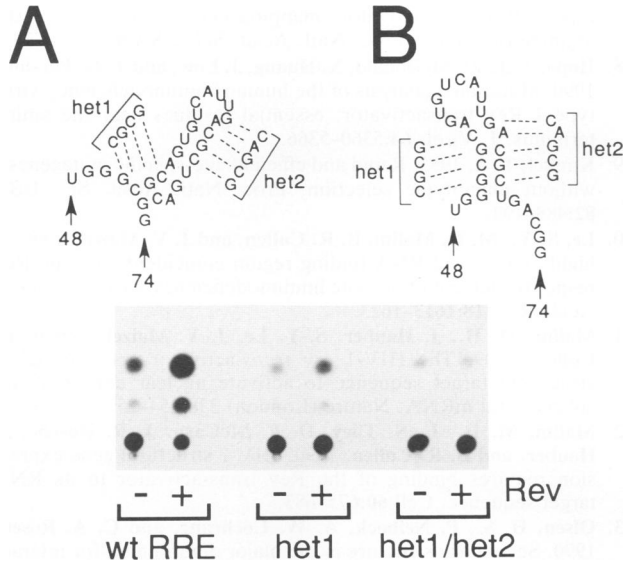

FIG. 2. Alternative models for the secondary structure of SL2A. The structures predicted by Malim et al. (11) (A) and Heaphy et al. (5) (B) for residues 48 to 74 of the RRE are shown (residues are numbered as described in the legend for Fig. 1B). Also shown are two sets of clustered point mutations (het1 and het2) introduced into this region of the 240-base RRE. Under model B, the het2 mutations would be expected to complement het1, restoring all putative base pairing while preserving an unpaired adenosine. Results of representative CAT assays of reporters containing the wild-type (wt) and mutant RREs are depicted below the models.

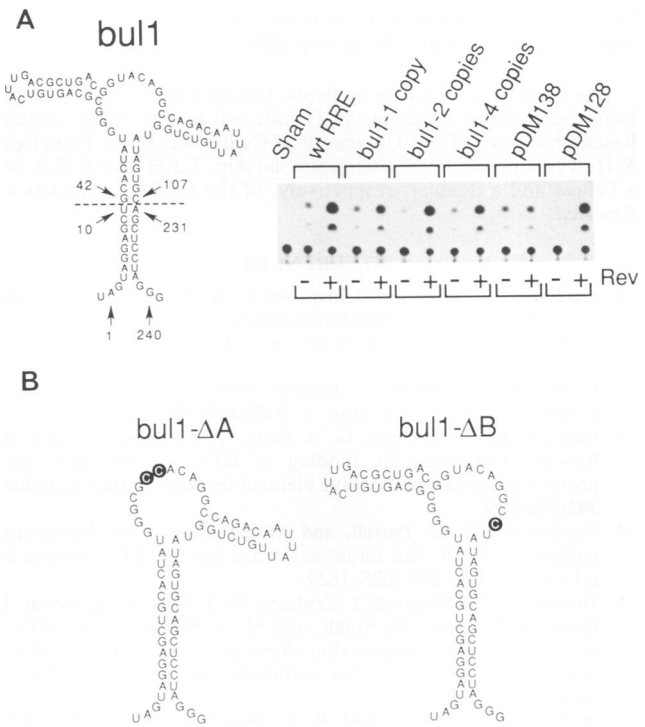

FIG. 3. An 88-base truncated RRE is sufficient for transactivation. (A) Sequence and predicted structure of the bul1 mutant RRE (numbered as described in the legend for Fig. 1B), with representative CAT assay results for pDM128, pDM138, and pDM138 derivatives containing either the 240-base RRE insert (wt RRE) or the indicated number of tandem copies of bul1. Lane Sham, Untransfected cells. DNA encoding the bul1 mutant was synthesized with two partially complementary oligonucleotides that together encompassed only the depicted sequence along with flanking *Cla*I sites; these oligonucleotides were annealed and extended with Klenow polymerase, trimmed with *Cla*I, and cloned in pUC118. Residues from stem 1 were included to promote proper folding; we have not yet determined whether they contribute to bul1 activity. (B) Two nonfunctional derivatives of the bul1 RRE. Each mutant was constructed from synthetic oligonucleotides as described above. Point mutations (open lettering) introduce a unique *Nar*I or *Stu*I site at the site of deletions in bul1-ΔA and bul1-ΔB, respectively.

The likely secondary structure of bul1 is depicted in Fig. 3A. When inserted into pDM138 and tested in the cotransfection assay, a single copy of bul1 yielded sixfold transactivation of CAT in response to Rev (Fig. 3A and Table 1). We found, moreover, that insertion of two tandem copies of bul1 was sufficient to yield reproducible 22-fold transactivation, a level of responsiveness fully comparable to that of the 240-base RRE. Curiously, four tandem copies of bul1 showed a degree of responsiveness similar to that observed with a single copy; this might reflect the increased likelihood that adjacent copies will anneal with one another to form aberrant, nonfunctional secondary structures. We subsequently tested two truncated forms of bul1, designated bul1-ΔA and bul1-ΔB, that lacked SL2A and SL2B, respectively (Fig. 3B). As indicated in Table 1, neither of these mutants was detectably responsive to Rev when present in a single copy.

Our study extends previous mapping of the essential features of the RRE. Although earlier reports have shown that SL2 is indispensable for function, mutations in other regions of the RRE (most notably in stem 1) have also been reported to inhibit Rev binding (1, 13, 16) and transactivation (4, 12, 13). This has prompted speculation that the latter regions might serve as binding sites for unidentified cellular factors that are needed for transactivation or that Rev binding might require two or more noncontiguous regions of the RRE (5, 13, 16). We have now demonstrated, however, that an 88-base truncated RRE (bul1) derived almost entirely from SL2 is sufficient to mediate sixfold Rev transactivation in vivo and that tandem copies of bul1 can impart a degree of responsiveness comparable to that of the full-length RRE. Sequences from the RRE that are not present in bul1 thus have no unique role in transactivation and make no more than a quantitative contribution to RRE function.

The SL2 sequence contained in bul1 is the minimal region known to bind Rev protein in vitro (5, 12). While this suggests that the ability to bind Rev may be the only requirement for RRE function, we cannot exclude the possibility that SL2 might also contain binding sites for unknown accessory factors or that Rev might act by displacing such factors from this region of the RRE. The finding that two tandem copies of bul1 are needed to fully replace the wild-type response element suggests that some regions in the RRE may be functionally redundant, perhaps serving as secondary sites for cooperative binding of Rev or other factors. For example, deletion of the SL2B arm completely inactivates bul1 but only partially inactivates the 240-base RRE (Table 1), suggesting that other sequences can compensate for the loss of SL2B in its normal context. The comparatively small size of bul1 may make it a useful model for

further investigation of these issues as well as for structural analysis of a biologically active RRE.

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