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Posttranscriptional Regulation by the Human Immunodeficiency Virus Type 1 Rev and Human T-Cell Leukemia Virus Type I Rex Proteins through a Heterologous RNA Binding Site

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The human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex proteins induce cytoplasmic expression of incompletely spliced viral mRNAs by binding to these mRNAs in the nucleus. Each protein binds a specific *cis*-acting element in its target RNAs. Both proteins also associate with nucleoli, but the significance of this association is uncertain because mutations that inactivate nucleolar localization signals in Rev or Rex also prevent RNA binding. Here we demonstrate that Rev and Rex can function when tethered to a heterologous RNA binding site by a bacteriophage protein. Under these conditions, cytoplasmic accumulation of unspliced RNA occurs without the viral response elements, mutations in the RNA binding domain of Rev do not inhibit function, and nucleolar localization can be shown to be unnecessary for the biological response.

The Rev protein of human immunodeficiency virus type 1 (HIV-1) and the Rex protein of human T-cell leukemia virus type I (HTLV-I) belong to a class of retrovirus-encoded nuclear proteins that control viral gene expression at the posttranscriptional level (reviewed in reference 9). Proteins of this type appear to act through a common pathway to induce accumulation of unspliced or incompletely spliced viral mRNAs in the cytoplasm (12, 14, 16, 17, 28, 31, 32). Though their exact mechanism of action is unknown, Rev and Rex have each been shown to bind directly and specifically to their responsive RNAs in vitro, as discussed below. It is thought that the interaction of Rev or Rex with viral precursor RNAs in the nucleus allows release of these RNAs to the cytoplasm before splicing is complete, perhaps by directly inhibiting splicing (5, 24, 25), by triggering premature RNA transport (28), or through some combination of these effects.

Rev and Rex have markedly dissimilar amino acid sequences but share a number of biological properties. Both are relatively small (116 and 295 amino acids, respectively) nuclear phosphoproteins that associate preferentially with nucleoli (14, 26, 35). Each acts selectively on RNAs that contain a specific *cis*-acting locus, known as the Rev response element (RRE) or Rex response element (XRE), respectively. The RRE and XRE each coincide with regions of complex RNA secondary structure and have been shown to contain high-affinity binding sites for the viral proteins. The RRE, for example, maps to a 240-base array of potential RNA hairpin loops (28) which can bind up to eight copies of Rev simultaneously in vitro (6, 8, 10, 18, 23, 29, 41), in part because Rev protein tends to oligomerize (27, 30, 42). In similar fashion, Rex binds specifically to the structured XRE, though the stoichiometry of this binding has not been determined (2, 4, 15, 17, 38).

Rev protein contains at least two essential functional domains. The larger N-terminal domain includes all of the sequences necessary for RRE binding, nuclear and nucleolar localization, and Rev protein oligomerization (7, 21, 26, 27, 30, 42). The second, more C-terminal region, here termed the effector domain, also is required for activity in vivo, but its exact function is unknown (26). Rex likewise contains an effector domain which is distinct from the sequences required for RNA binding and for nuclear and nucleolar localization (19, 31, 40), and there is evidence that Rev-like proteins from other retroviruses may be organized in the same manner (37). Significantly, the effector domains of Rev, Rex, and at least one other protein of this type are functionally interchangeable and share certain degenerate sequence motifs that are essential for activity (19, 37, 40). This finding suggests that all such effector domains may subserve a common function, perhaps mediating regulatory interactions with components of the cellular RNA splicing or transport apparatus.

Binding of Rev or Rex proteins to the viral response elements is critical for their biological effects. When coupled with specific splice site mutations that decrease the efficiency of splicing, insertion of the RRE is sufficient to confer Rev responsiveness onto a cellular RNA (5). Rev binding appears to be accompanied by conformational changes in both the protein and the RRE (11, 23), but it is not known whether these changes are important for triggering the response. It also is not known whether cellular factors also must interact with the RRE or XRE, or whether these elements make other unrecognized contributions to the response. The fact that both Rev and Rex tend to localize in nucleoli suggests that this organelle might have an important role in the response, but it has been difficult to evaluate the significance of this localization conclusively because mutations that inactivate the nucleolar localization signals in Rev or Rex (7, 20, 28, 31, 35) also abolish RNA binding (4, 27, 30, 42).

To dissect the roles of these retroviral proteins and their RNA response elements, we have created a series of chimeric proteins in which Rev and Rex are fused to the coat protein of bacteriophage MS2. The MS2 coat protein has no intrinsic regulatory activity in eukaryotic cells but binds as a dimer to a specific 21-base RNA operator (references 33, 34, and 39 and references therein). In this report, we demonstrate that Rev and Rex can exert their effects when tethered

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by this phage protein to a target RNA containing the MS2 operator. Under these conditions, nucleolar localization, the viral RNA response elements, and the specific RNA binding sequences in Rev can all be shown to be unnecessary for inducing release of unspliced RNAs to the cytoplasm.

MATERIALS AND METHODS

Plasmid construction. The reporter pDM138, as well as derivatives containing the RRE, XRE, or bul1 sequences, have been described previously (19, 22). Additional response elements were synthesized as overlapping oligonucleotides with flanking *Cla*I sites and were ligated into the *Cla*I site of pDM138. The reporter used for Northern (RNA) blot experiments was derived from pDM138 by inserting MS2.4 and replacing the simian virus 40 promoter with the cytomegalovirus immediate-early gene promoter; this variant yielded higher levels of mRNA expression than does its simian virus 40 counterpart but gave proportionally similar chloramphenicol acetyltransferase (CAT) responses (data not shown). Wild-type and mutant forms of the expression plasmids pRSV-Rev and pRSV-Rex have also been described (19–22); to create fusion proteins, the last *rev* or *rex* codon in these plasmids was converted to a *Bgl*II site (encoding Asp-Leu) by oligonucleotide-directed mutagenesis, and a polymerase chain reaction fragment comprising codons 1 to 130 of MS2 coat protein was then inserted in frame. All fusions and mutations were confirmed by DNA sequencing.

Protein expression assays. For CAT enzyme assays, CV1 cells were transfected by the calcium phosphate method with a transactivator expression plasmid, a reporter containing the appropriate response element, 0.25 μg of β-galactosidase vector pDM110, and sufficient pUC118 for 10 μg of total DNA. CAT activity was assayed by thin-layer chromatography after normalization to β-galactosidase expression and was quantified by scintillation counting; details of this assay have been reported elsewhere (20, 21). Immunoblots and in situ immunostaining were performed as described previously (21).

RNA expression assay. For isolation of cytoplasmic RNA, CV1 cells from each transfected 10-cm^2 plate were washed once in Tris-buffered saline and then resuspended in 200 μl of ice-cold 10 mM Tris (pH 8.0)–10 mM NaCl–1.5 mM $MgCl_2$–0.5% (wt/vol) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS)–20% (wt/vol) sucrose. Nuclei were removed by microcentrifugation (1 min and then 10 min) at 4°C. Supernatants were combined with 200 μl of Tris buffered saline–0.5% CHAPS–0.5% sodium dodecyl sulfate and repeatedly extracted with phenol-chloroform. RNA was then precipitated with ethanol, analyzed by formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose, and probed for a region of the HIV-1 long terminal repeats found in reporter transcripts but not in pRSV-Rev (20).

RESULTS

Much of the 240-base RRE can be deleted without eliminating either Rev protein binding in vitro or Rev responsiveness in vivo; however, a discrete 77-base region known as stem-loop 2, which serves as the primary Rev binding site in the RRE, has been found to be both necessary and sufficient for biological activity (reference 22 and references therein). This finding suggests that Rev binding might be the sole requirement for RRE function. To test this hypothesis, we modified the mammalian Rev expression vector pRSV-Rev

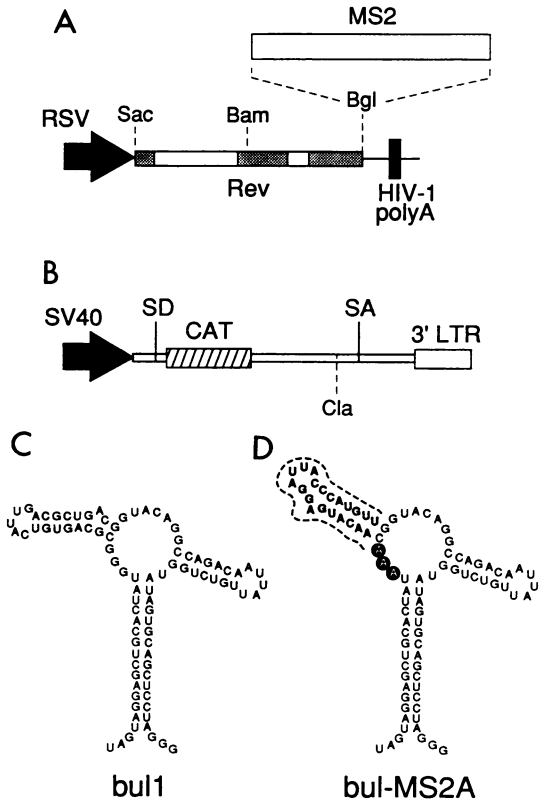

FIG. 1. Reporters and RRE derivatives for analysis of the Rev response. (A) Structure of pRSV-Rev, which expresses HIV-1 Rev cDNA by using the Rous sarcoma virus (RSV) promoter and HIV-1 polyadenylation signals (20). Bam, *Bam*HI; Bgl, *Bgl*II; Sac, *Sac*I. (B) Structure of reporter pDM138, which contains the simian virus 40 (SV40) early promoter, HIV-1 *rev* splice donor (SD) and acceptor (SA) sites, 3′ HIV-1 long terminal repeat (LTR), the CAT coding sequence, and a *Cla*I (Cla) linker. (C) Sequence and putative structure of bul1, a functional response element (22) encompassing stem-loop 2 of the RRE (29). (D) Sequence and possible structure of the bul-MS2A variant of bul1, with MS2 operator (outlined) and three point mutations (open lettering) indicated. Response elements were ligated into the *Cla*I site of pDM138.

to encode a chimeric protein (Rev/MS2) in which the MS2 phage coat protein is fused to the C terminus of Rev (Fig. 1A). We reasoned that fusion with the coat protein would enable Rev to bind indirectly to target RNAs containing the MS2 operator and that such indirect binding might support function (34).

Rev activity can be assayed (22) by inserting various forms of the RRE into the CAT reporter plasmid pDM138 (Fig. 1B). When transfected transiently into CV1 cells, the resulting constructs generate transcripts containing both the CAT coding sequence and the inserted RRE within a single intron flanked by HIV-1 splice sites. The unspliced reporter transcripts are exported to the cytoplasm in a Rev-dependent manner (20); thus, CAT enzyme expression is markedly increased by cotransfection of pRSV-Rev. The minimal sequence known to confer Rev responsiveness onto pDM138 is an 88-base element called bul1 (Fig. 1C), which encompasses the entire stem-loop 2 region of the RRE (22). For this study, we eliminated sequences from bul1 that are essential for Rev binding and replaced them with the MS2 operator, producing a modified response element called bul-MS2A

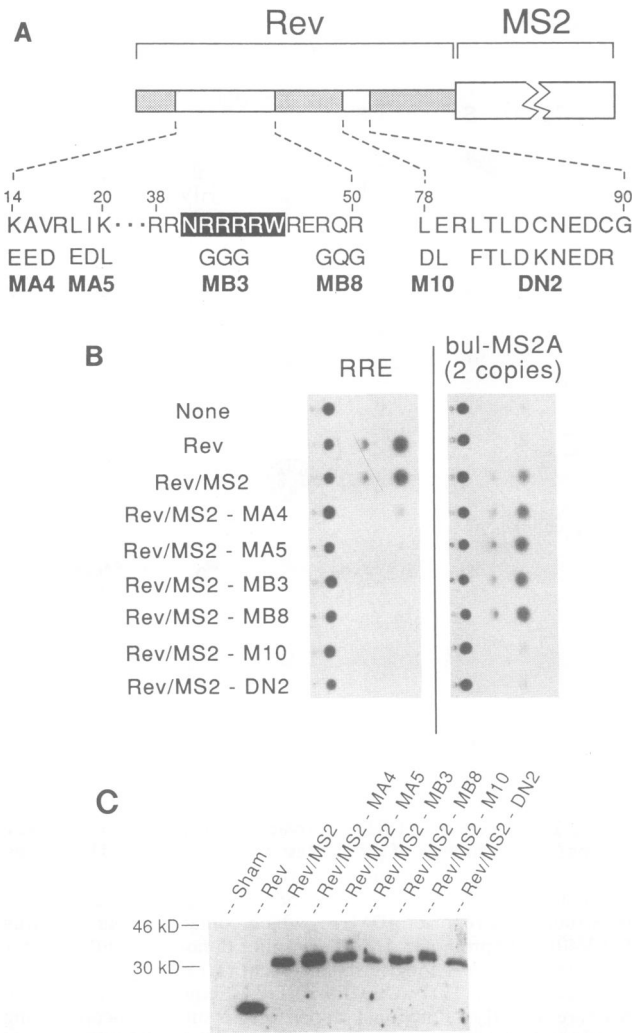

FIG. 2. Functioning of the Rev/MS2 fusion protein through a modified response element. (A and B) Structures and activities of wild-type and mutant forms of Rev/MS2. (A) Schematic view of Rev/MS2. Open rectangles in the Rev moiety represent the essential N-terminal and effector domains; partial sequences of each are shown, along with the six mutations tested in this study. Open lettering indicates a hexapeptide that is required for nuclear and nucleolar localization (7, 20). (B) CAT expression in CV1 cells 40 h after transfection with a pRSV-Rev derivative (4 μg) encoding the indicated proteins, a reporter (1 μg) containing either the full-length RRE or two tandem copies of bul-MS2A, 0.25 μg of pDM110, and sufficient pUC118 for 10 μg of total DNA. CAT activity was assayed after normalization to β-galactosidase expression. The RRE reporter was pDM128, which yields 100-fold CAT induction with pRSV-Rev (20). None, reporter alone. (C) Immunoblot analysis of mutant proteins. Positions of molecular weight standards are shown at the left; unfused MS2 coat protein is 13.7 kDa.

(Fig. 1D). Because earlier studies had shown that duplicate copies of bul1 are needed for maximal (23-fold) CAT induction by Rev in this context (22), we initially inserted two tandem copies of bul-MS2A into pDM138.

When tested in the cotransfection assay (Fig. 2B), Rev/MS2 induced CAT expression through the full-length RRE as efficiently as did Rev. Unlike Rev, however, Rev/MS2 also functioned through bul-MS2A, implying that fusion with the MS2 coat protein could extend the target range of Rev. To confirm that Rev protein sequences were necessary for this response, we introduced a series of missense mutations into the two functional domains in the Rev moiety (Fig. 2A). Each of the mutations that we tested had previously been shown to abolish Rev function through the RRE (20, 21). We found that four separate mutations involving the N-terminal domain (MA4, MA5, MB3, and MB8), each of which eliminates the RRE binding and oligomerization activities of Rev in vitro (42), eliminated Rev/MS2 function through the RRE but not through bul-MS2A. In contrast, two different mutations (M10 and DN2) that have been shown to inactivate the Rev effector domain (21, 26) each abolished function through both response elements. Immunoblots confirmed the sizes and stabilities of all six mutant proteins (Fig. 2C). Thus, transactivation through bul-MS2A required the coat protein in conjunction with an intact Rev effector domain but was not inhibited by mutations that eliminate RRE binding.

Mutation MB3 was of particular interest, as it is known also to prevent nucleolar localization of Rev (20). Using in situ immunofluorescence, we found that wild-type Rev/MS2 was expressed throughout the nucleoplasm of transfected cells and was especially abundant in nucleoli (Fig. 3A); significant amounts the protein were also detected in the cytoplasm, suggesting that Rev/MS2 may be translocated into the nucleus somewhat less efficiently than Rev. In contrast, the mutant Rev/MS2-MB3 also was found throughout the nucleoplasm and cytoplasm but was selectively excluded from nucleoli (Fig. 3B). Nevertheless, this mutant functioned at least as efficiently as Rev/MS2 through bul-MS2A (Fig. 2B; see below). Preferential nucleolar localization is therefore not essential for Rev/MS2 activity.

These observations were extended in tests of a similar fusion of the MS2 protein with HTLV-I Rex (Table 1). Rex contains an effector domain that is functionally interchangeable with that of Rev (19, 40), but the two proteins have significantly different RNA binding specificities; in particular, Rex cannot interact functionally with the portion of the RRE contained in bul1 (1, 4). We found, however, that Rex/MS2 functioned almost as efficiently through two copies of bul-MS2A as it did through the XRE and that a mutation (M510) known to inactivate the Rex effector domain (19) eliminated this response. Thus, fusion with coat protein extended the target ranges of both Rev and Rex, but only if their effector domains were intact.

To determine whether the MS2 operator alone could mediate the response to these fusion proteins, we then designed three completely heterologous elements (Fig. 4A) that included no XRE or RRE sequences but contained one, two, or four operators, respectively. Each was inserted into pDM138 and tested for responsiveness to Rex/MS2 and the Rev/MS2-MB3 mutant (Fig. 4B). We found that reporters containing only one operator (MS2.1 or a single copy of bul-MS2A) gave little or no response, but that those containing two or more operators (MS2.2 and MS2.4) responded strongly to both fusion proteins. Indeed, the CAT responses achieved by Rex/MS2 through MS2.4 were equal in magnitude to those it produced through the XRE (Table 1). The response thus required no XRE or RRE sequences, provided that two or more MS2 operators were present.

Northern blots of cytoplasmic RNA from the transfected cells confirmed that the CAT inductions that we had observed were due to changes in mRNA expression (Fig. 5). We found that CV1 cells transfected with an MS2.4-containing derivative of pDM138 expressed spliced reporter transcripts constitutively in the cytoplasm. Cotransfection with a Rex/MS2 expression vector reproducibly led to the accumu-

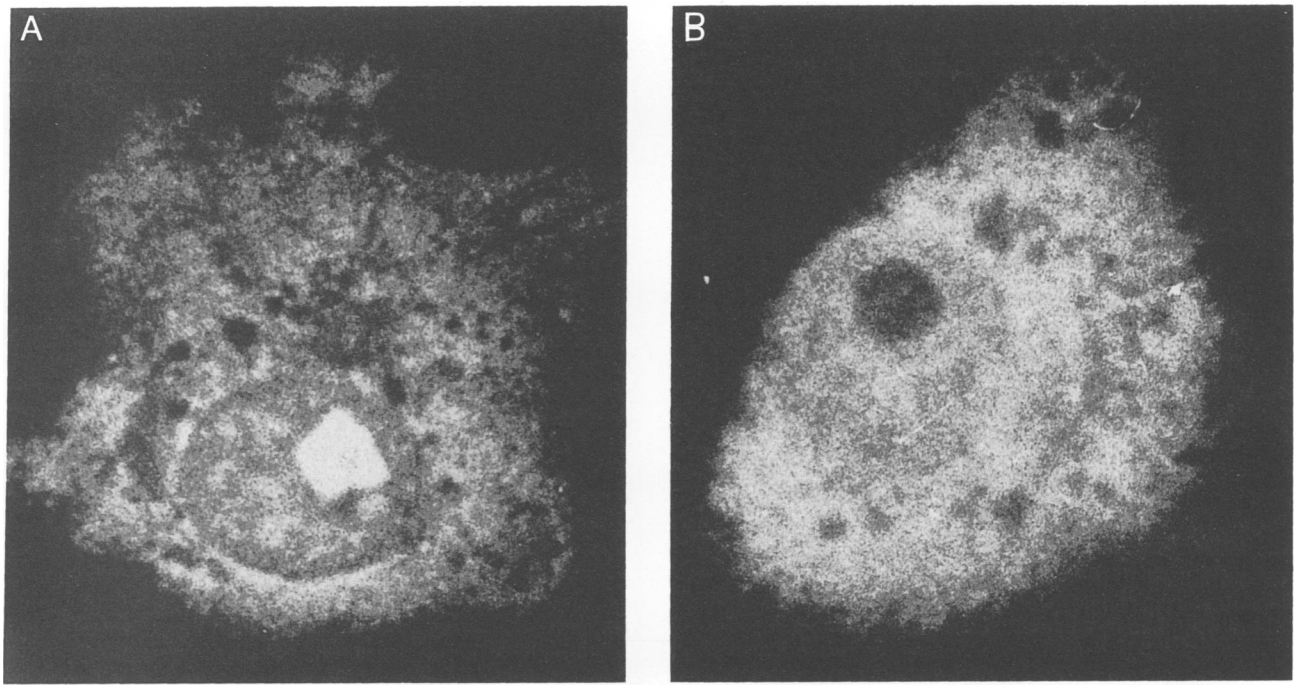

FIG. 3. A functional Rev derivative with defective nucleolar localization. In situ immunolocalization of Rev/MS2 (A) and Rev/MS2-MB3 (B) was performed in transfected COS7 cells, using an antiserum specific for the C-terminal half of Rev. The patterns shown typified essentially all immunoreactive cells in each population.

lation of unspliced reporter transcripts in the cytoplasm, and the M510 effector domain mutation completely eliminated this response.

An arginine-rich peptide from the Rev N-terminal domain has been reported to inhibit splicing of RRE-containing RNAs in vitro (24). We found that while much of the N-terminal domain could be deleted from Rev/MS2 without inhibiting function through MS2.4, selective deletion of the arginine-rich sequence completely inactivated the protein (Fig. 6). Activity was fully restored, however, when we substituted a different arginine-rich sequence (Scram) that lacks the ability to inhibit splicing in the in vitro assay (24). This finding implies that while basic amino acids may be required at this location in the fusion protein (perhaps to strengthen RNA binding through nonspecific contacts), the arginine-rich domain of Rev makes no sequence-specific contribution to the responses that we observed.

TABLE 1. Functioning of Rex/MS2 fusion protein through the bul-MS2A response element

Response element	Transactivator	CAT activity (% acetylation)[a]	Fold induction
XRE	None (reporter alone)	0.9 ± 0.1	
	Rex	54.3 ± 0.4	60
	Rex/MS2	25.2 ± 2.5	28
	Rex/MS2-M510	1.1 ± 0.1	1
Bul-MS2A (2 copies)	None	1.3 ± 0.1	
	Rex	1.0 ± 0.1	1
	Rex/MS2	31.1 ± 0.6	24
	Rex/MS2-M510	1.2 ± 0.1	1

[a] In CV1 cells transfected with a pDM138 derivative containing either a single XRE or two copies of bul-MS2A, along with plasmids encoding the indicated transactivators. Data are means ± standard errors of the means from triplicate transfections, assayed as described for Fig. 2. Mutation M510 replaces Rex residues 90 to 93 with glycines (19). The stability of Rex/MS2-M510 was confirmed by immunoblot (data not shown).

DISCUSSION

The results of this study reveal that fusion with a bacteriophage RNA-binding protein can redirect the target specificities of Rev and Rex in vivo. This finding confirms the strong inference from earlier data that RNA binding is critical for the response to these proteins (2, 4, 6, 10, 15, 18, 29, 36, 41). Our data also shed new light on the minimal requirements for this response. Most notably, we find that when tethered to RNA by a bacteriophage protein, Rev and Rex can each induce cytoplasmic expression of intron-containing RNAs which contain no RRE or XRE sequences. The responses achieved under these conditions (by using the heterologous target element MS2.4) are 35 to 50% as strong as those produced by unfused Rev or Rex through their native viral response elements. Thus, at least a substantial proportion of the response does not depend upon any unique structural features of the RRE or XRE or on recognition of these elements by cellular proteins, except insofar as these elements might be required for binding a particular transactivator (3). Although other specific properties of the viral elements (24) might be needed for a maximal quantitative response, the ability to bind Rev or Rex is the only essential requirement for RRE or XRE function in vivo.

Rev and Rex each contain sequences that function as nucleolar localization signals (7, 20, 31, 35). Early reports showed that mutations in these sequences profoundly inhibit transactivation, but the subsequent finding that these mutations also prevent RNA binding has left the significance of this localization unresolved (2, 4, 15, 27, 30, 42). Our studies

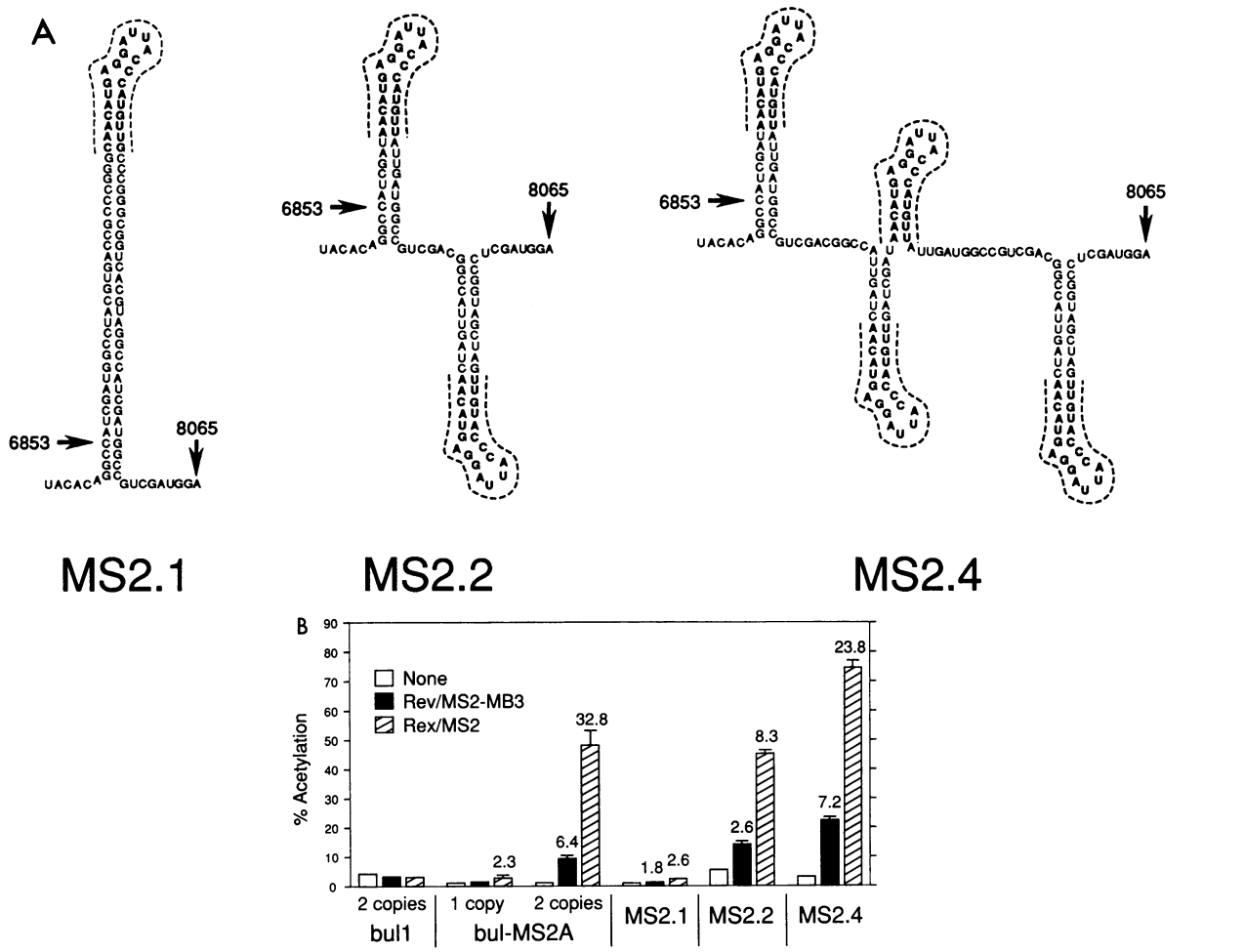

FIG. 4. Functioning of Rev and Rex fusion proteins in the absence of the viral response elements. (A) Sequences and possible structures of heterologous elements containing the MS2 operator (outlined). Each is shown as an insertion between HIV-1 residues 6853 and 8065 (arrows) in the pDM138 transcript. MS2.4 is two-tandem copies of MS2.2; lower-energy conformations are possible. (B) Functional testing of heterologous elements. CV1 cells were transfected with pDM138 containing the indicated response elements, either alone (None) or with a plasmid encoding Rex/MS2 or Rev/MS2-MB3. CAT expression (relative to that of a β-galactosidase control minus the activity of sham-transfected cells) in triplicate transfections was assayed at 36 h and quantified by scintillation counting. Error bars indicate standard errors of the means SEM exceeding ±0.4% acetylation; numbers above bars indicate responses expressed as fold CAT induction.

now demonstrate that when RNA binding is maintained by the phage protein, nucleolar localization is not required for Rev function. This finding suggests that the localization of Rev and Rex simply reflects their affinity for nucleolar constituents that are not required for the response, a view supported by recent in vitro evidence of Rev's interactions with nucleolar proteins (13).

The effector domains from Rev and Rex proved essential and interchangeable for function of our fusion constructs, as they are in the native proteins. By contrast, mutations throughout the N-terminal Rev domain did not diminish function through the phage operator, implying that sequence-specific contacts between this domain and the target RNA are not needed for the responses that we observed. Moreover, as the N-terminal mutations that we tested also inactivate the oligomerization signals in Rev (42), oligomerization per se also does not appear to be essential. This finding weighs against the possibility that oligomerization of Rev through its N terminus might be required for allosteric activation of the effector domain.

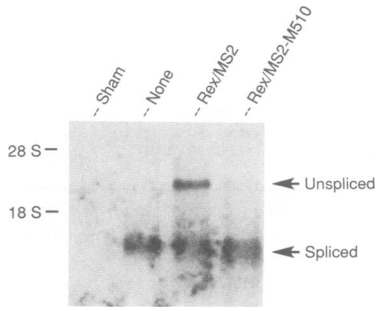

FIG. 5. Northern blot detection of reporter transcripts in cytoplasmic RNA. CV1 cells were transfected with the MS2.4-containing reporter (10 μg) either alone (None) or along with a vector encoding Rex/MS2 or Rex/MS2-M510 (5 μg). Total cytoplasmic RNA was isolated 48 h after transfection, and 5-μg samples were analyzed on a 1% agarose–formaldehyde gel and probed for HIV-1 long terminal repeat sequences not present in pRSV-Rev. Positions of the 18S and 28S rRNAs are indicated. Sham, 10 μg of pUC118 alone.

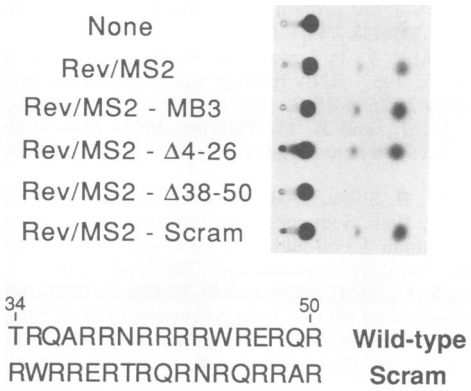

FIG. 6. Mutagenesis of the N-terminal domain in Rev/MS2. CV1 cells received plasmids encoding the indicated Rev/MS2 variant together with a pDM138 derivative containing MS2.4. Samples were assayed as described in the legend to Fig. 2A. Δ, deletion of residues from Rev; Scram, replacement of residues 34 to 50 with the sequence shown. Properties of the Scram peptide have been described elsewhere (24). None, reporter alone. Rev/MS2-Scram does not function through the RRE (data not shown).

Our findings strongly suggest, moreover, that multiple effector domains must be linked to each target RNA in order to trigger a response: assuming that all operator sites are accessible and competent for binding and that the fusion proteins bind as MS2 dimers (39), the data in Fig. 3B suggest a threshold requirement for three or four effector domains per transcript. This possibility is consistent with reports (8, 10, 23) that a single RRE can bind up to eight copies of Rev and that a single bound Rev is not sufficient for function (27). Our data suggest the further conjecture that HTLV-I transactivation may require binding of multiple copies of Rex to the XRE.

Taken together, these studies suggest a model in which the effect of Rev or Rex on RNA localization depends upon the linkage of a critical number of effector domains to a target RNA. For this aspect of transactivation, the remaining portions of each protein, and the viral RNA response elements themselves, serve only to facilitate such linkage and are potentially dispensable. The nucleolar localization of Rev and Rex can be viewed as reflecting their affinity for nucleolar constitutents that have no obligatory role in releasing unspliced viral mRNAs from the nucleus.

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