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Human Immunodeficiency Virus Type 1 Rev Activation Can Be Achieved without Rev-Responsive Element RNA if Rev Is Directed to the Target as a Rev/MS2 Fusion Protein Which Tethers the MS2 Operator RNA

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The posttranscriptional *trans* activation of unspliced or partially spliced human immunodeficiency virus RNAs by the Rev regulatory protein is crucial for virus replication and is dependent on sequence-specific RNA binding by Rev. The cognate RNA target of Rev is contained within a highly structured, 244-nucleotide Rev-responsive element (RRE) RNA in the viral *env* gene. Here, we show that specific interaction with the RRE is not an absolute requirement for Rev function. When the RRE is replaced by a heterologous MS2 phage operator sequence, Rev will facilitate the cytoplasmic expression of human immunodeficiency virus mRNAs containing this sequence if directed to the MS2 operator via the RNA binding motif of the MS2 phage coat protein (MS-C) as a Rev/MS-C fusion protein. Rev/MS-C efficiently activated both RRE and MS2 targets. A mutation in the MS2 operator that abolished the coat protein binding in vitro rendered the mutant RNA nonresponsive to the fusion protein in vivo. Notwithstanding that Rev can be tethered to the viral RNAs via another RNA binding motif, the structural integrity of the N terminus of Rev was still required for optimal *trans* activation.

The life cycle of human immunodeficiency virus type 1 (HIV-1) includes several biochemical mechanisms that may also be general paradigms for eukaryotic gene regulation. Sequence-specific interaction of viral RNAs with virus-encoded proteins is a common theme in many fundamental viral processes, such as RNA transcription and posttranscriptional activation and RNA encapsidation (13, 45). HIV-1 transcription is activated by the viral Tat protein, which recognizes a 22-nucleotide stem-loop nascent TAR RNA (7, 9, 21, 42, 46). HIV Rev acts posttranscriptionally to modulate the splicing (11, 30) and to facilitate the extranuclear transport and cytoplasmic utilization of viral mRNAs by binding to a highly structured Rev-responsive element (RRE) sequence in the *env* mRNA (15–17, 19, 20, 23, 32, 35, 49). Rev is a basic protein that concentrates in the nucleoli (12, 14) and readily oligomerizes both in vitro and in vivo (33, 36, 47, 50). Genetic studies have identified separate nuclear localization-RNA binding and activation domains at the N- and C-terminal portions of the Rev protein, respectively (31). Genetic analyses of the N-terminal region of Rev have mapped a contiguous stretch of amino acid residues between positions 13 and 56 that confers RNA binding, nucleolar localization, and oligomerization properties essential for Rev function (5, 8, 27, 33).

Interest in the specific sites of functional contact of Rev with the RRE has led to attempts to narrow the element down to a minimal responsive motif. The computer-predicted secondary structure of RRE RNA (32) is composed of four stem-loops (A, C, D, and E) and a branched stem-loop (B/B1/B2) surrounding a central loop sequence (Fig. 1). Large portions of this RRE structure are dispensable for the Rev response (17, 22, 23, 35), and a 64-nucleotide subsequence, predicted to fold into the branched stem-loop B/B1/B2, retains the Rev response both in vitro and in vivo (23, 35). A 9-nucleotide 5′-CACUAUGGG-3′ sequence of the RRE corresponding to the B/B1 regions represents the minimal Rev recognition sequence, both in vitro and in vivo, if it is presented as a stem-bulge-stem structure and contains at least two G's, one of which must be unpaired (24).

In spite of the accumulated evidence of the role of the RRE as an anchor for Rev, the possibility that the RRE also recruits cellular factors that may be agonists or antagonists of Rev function cannot be excluded. For instance, Rev cannot activate the 5′-most 132-nucleotide RRE fragment in vivo, despite having a greater in vitro affinity for this fragment than the full RRE (23). It is possible that this 132-nucleotide fragment lacks an essential recognition motif for a putative cellular helper factor(s). In fact, a 56-kDa RRE-binding cellular factor that may assist in Rev function has been demonstrated previously (44). Also, both stably and transiently expressed Rev functions poorly in murine cells, implying a requirement for tissue- or species-specific Rev helper factors (43).

To explore the role of the RRE in the *trans*-activation processes other than Rev binding and to examine the contributions of the peptide motifs of Rev to activation, we have devised a functional assay for Rev with heterologous RNA targets. We show that the RRE may be replaced by the heterologous MS2 phage operator RNA without losing the Rev response in vivo if Rev is directed to the operator as a Rev/MS2 coat protein fusion. Using bivalent targets recognized by both Rev and the MS2 coat protein, we demonstrate that the in vivo responses of the targets parallel their in vitro affinities for the respective RNA binding proteins. These studies indicate that Rev binding may be the primary function of the RRE and that RRE-binding cellular factors may not be an absolute requirement for the *trans*-activation effect of Rev.

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MATERIALS AND METHODS

***gag* expression plasmids.** All of the recombinants were constructed by site-directed mutagenesis of RRE DNA with a commercial M13-based protocol (Mutagene kit; Bio-Rad Laboratories, Richmond, Calif.), and the respective mutant DNAs were exchanged for the RRE in the HIV-1 long terminal repeat (LTR)-linked *gag* expression vector containing the wild-type (wt) RRE (23). RREZ-MS was constructed by first deleting the Rev-responsive B/B1/B2 subdomain (23) in the RRE (RRE coordinates 49 to 113) and then inserting a modified version of phage MS2 translational operator sequence (6). RREZ-MS in M13 was further mutagenized to generate RREZ-MSΔA, RREZ-MS(U/A), and RREZ-MS(A/C), which refer to the mutants which deleted the bulged A residue (underlined in the -GAGG- sequence) or substituted A for U(-AUUA-) or C for A (-AUUA-) in the loop sequence of MS. In addition, the RRE was also completely exchanged for a monomeric MS sequence in the sense (MS) or antisense (SM) orientation and for a head-to-tail [(MS)(MS)] or head-to-head [(MS)(SM)] dimer of the modified operator. (MS) was constructed by inserting the modified operator flanked by two A residues at the Asp-718 (*Kpn*I) site of M13mp18, and the MS monomer was subsequently exchanged for the RRE in the wt *gag* RRE plasmid. To generate [(MS)(SM)] and [(MS)(SM)], the MS monomer was recovered from the recombinant M13 replicative form by Asp-718 digestion and self-ligated. The MS dimer from this reaction was recovered by polyacrylamide gel electrophoresis and inserted into the above *gag* expression plasmid in place of the RRE. (MS)+A was constructed by inserting the RRE sequence corresponding to positions 31 to 49 and positions 204 to 221, upstream and downstream, respectively, of the MS sequence in the M13 recombinant. In (MS)+a, RRE sequence between positions 39 to 49 and 204 to 213 flanked the MS sequence. The following two chimeras incorporating the targets for both Rev and the MS coat protein were also engineered. (i) To construct (MS)+B/B1/B2, the B/B1/B2 subdomain (positions 49 to 115) of the RRE was initially cloned into M13mp18. MS sequence flanked by three A's was then inserted upstream of the B/B1/B2 motif. (ii) M13 containing B/B1/B2 was also mutagenized to replace the B1 stem-loop (positions 59 to 82) with the MS2 sequence. The resulting recombinant B/3G/MS2/B2 preserved the B stem and contained the RRE recognition sequence -CACUAUGGG- upstream of the operator. Each of the bivalent chimeras was individually mutagenized to substitute the three G's in the RRE B stem with three A's, to delete the bulged A residue in the MS operator, or to do both. All of the chimeras were then inserted into the *gag* expression plasmid pRMK4RRE in place of the RRE.

Expression plasmids for Rev/MS-C fusion proteins and their derivatives. HIV-1 Rev was expressed either from the Tat-responsive HIV-1 LTR or from the constitutive Rous sarcoma virus (RSV) LTR (29). MS-C and MS-N were expressed from the pSRα promoter (6). Rev/MS-C denotes a tandem fusion of Rev and MS-C open reading frames (ORFs). The fused gene was constructed by a two-step polymerase chain reaction (PCR) procedure. In the first step, the Rev ORF was PCR amplified with an *Xba*I restriction site-tagged 5′ oligonucleotide primer and a 3′ primer that was tagged with sequences corresponding to codons 2 through 7 of MS-C, MS-D, or MS-N. The MS2 coat protein expression plasmids were generously provided by Benjamin Berkhout of the University of Amsterdam, The Netherlands. The ORFs for the respective MS2 coat proteins were PCR amplified with a 5′ primer tagged with a sequence corresponding to the C-terminal 6 codons of Rev and a 3′ primer tagged with an *Xba*I site. The PCR-amplified Rev and MS DNAs were gel purified, annealed together, and amplified further with *Xba*I restriction site-tagged 5′ primers complementary to the codons at the N terminus of Rev and the C terminus of MS, respectively. The resulting Rev/MS fusion genes were purified from gels, cleaved with *Xba*I, and cloned into M13mp18. After the DNA sequence of the Rev/MS-mp18 recombinants was verified, the fusion gene was PCR amplified by primer pairs tagged with T7 phage RNA polymerase promoter and terminator sequences at the 5′ and 3′ ends, respectively. The resulting T7 template was transcribed in vitro with T7 RNA polymerase, and the RNA was translated in a rabbit reticulocyte translation system with [^{35}S]methionine. The translation yielded a fusion protein with the expected molecular mass that was readily immunoprecipitable with anti-Rev antiserum. All other Rev/MS-C derivatives carrying the indicated mutations were constructed by site-directed mutagenesis of Rev/MS-C M13mp18 phage DNA according to a commercial protocol (Mutagene kit, Bio-Rad Laboratories). All of the Rev/MS chimeras were cloned at the *Xba*I site of an RSV LTR-linked eukaryotic expression plasmid, pRSV.5 (29).

***Escherichia coli* expression of Rev/MS-C fusion proteins.** Rev/MS-C fusion protein and the indicated mutants were expressed in *E. coli* as fusion proteins linked to the C terminus of *E. coli* maltose-binding protein (MBP) from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible β-galactosidase promoter with a commercial kit (New England Biolabs, Beverly, Mass.). Rev/MS-C and Rev(LE/PE)/MS-C mutant ORFs were recovered from the respective RSV LTR-linked eukaryotic expression plasmids by *Xba*I digestion and cloned downstream of the lone *Xba*I site in the MBP vector. As a negative control, an Rev/MS-C coding sequence was also cloned in the antisense polarity with respect to the MBP ORF. Biosynthesis of the MBP-fused proteins was induced by IPTG (1 mM) at an optical density at 600 nm of 0.3 followed by 2 h of incubation in at 37°C. Bacterial cells were harvested, rinsed with phosphate-buffered saline (PBS), and resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted with a French press at 10,000 lb/in^2 and centrifuged at 25,000 × *g* for 15 min to collect the pellet containing the inclusion bodies. The pellet was completely dissolved in Tris buffer containing 50 mM Tris-HCl, 50 mM KCl, and 5 mM EDTA supplemented with 6 M guanidine HCl and dialyzed successively against 200 volumes of the above buffer containing 1, 0.5, and 0.05 M guanidine HCl for 4 h at each step. Samples were centrifuged at 10,000 × *g*, and the supernatant was stored at −70°C or directly bound to amylose resin under conditions recommended by the manufacturer. The fusion protein was eluted with 20 mM maltose, concentrated by ultrafiltration, and used for binding studies.

RNA synthesis and protein binding. DNA templates, tagged at their 5′ ends with the T7 promoter, were generated by PCR with primer pairs corresponding to the ends of the desired RNA (23). T7 promoter-tagged, PCR-amplified DNA fragments (0.5 pmol) were used as templates to generate unlabeled or uniformly labeled ([α-^{32}P]UMP) RRE transcripts with a commercial kit from Promega Biotec (Milwaukee, Wis.). All of the RNAs were purified by gel filtration on Sephadex G-100 columns and then electrophoresed on urea-acrylamide gel (10%), if necessary. RNA-protein binding

was evaluated by RNA gel mobility shift experiments. A reaction mixture containing protein samples and heparin (5 μg per reaction mixture) in HEPES binding buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-KOH, pH 7.9, 62 mM KCl, 0.15 mM dithiothreitol, 6% glycerol) was preincubated at 30°C for 10 min before the labeled RNA (5×10^3 cpm) was added and further incubated for 10 min at 30°C. Samples were loaded onto 5% native polyacrylamide gel in 0.5× Tris-borate-EDTA and electrophoresed at 30 mA at 4°C. Radioactivity was visualized by autoradiography of dried gels. Rev used in these experiments was expressed in *E. coli* and purified to near homogeneity (47).

Transient expression assay. Although the results presented in this article were obtained with HeLa cells, all transfections were repeated four times with Cos-7 cells. Approximately 2×10^6 HeLa or Cos-7 cells were electroporated at 300 V and 250 μF in a Bio-Rad electroporator with the individual *gag* plasmids (5 μg) and 2 μg of pHIV-Tat (pSV40 Tat for Cos-7 cells) and with or without the indicated Rev or the Rev/MS coat protein fusion protein expression plasmids. For the expression of Rev, 2.5 or 5 μg of an HIV-1 LTR-linked (23) or an RSV LTR-linked plasmid was used. The Rev/MS fusion protein plasmids (5 μg per experiment) were expressed from the RSV LTR. All transfections also included HIV-1 LTR-linked chloramphenicol acetyltransferase (CAT) (2 μg) plasmid to monitor the transfection efficiency. Aliquots of transfected cells were routinely removed at 48 h and used for immunodetection of Rev and Rev/MS fusion proteins (see below). Alternatively, aliquots of cells from each transfection were plated on coverslips immediately after electroporation and processed for immunofluorescence of Rev and Rev/MS fusion proteins. Transfections were routinely terminated at 48 to 60 h, the cells were disrupted by three freeze-thaw cycles, and the extracts were clarified by low-speed centrifugation. *gag* expression was quantitated by a p24 enzyme-linked immunosorbent assay (ELISA) of cell extracts (Coulter Diagnostics). $p24^{gag}$ expression levels were normalized to constant values of CAT activity expressed from HIV LTR-linked CAT (23). Each *gag* ELISA value from HeLa cell transfections represented a mean from at least eight independent transfections with multiple preparations of plasmid DNA. CAT expression was quantitated by a commercial CAT ELISA kit (Boehringer Mannheim).

Immunoblotting. After electroporation, cells were plated on 60-mm-diameter dishes and harvested 48 to 72 h later. A one-third equivalent of cells from each dish was collected by centrifugation, rinsed twice in PBS, and lysed by two freeze-thaw cycles in 50 μl of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, and 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate. The cell lysates were then adjusted to 100 μM phenylmethylsulfonyl fluoride–1 μg of aprotinin per ml–1 μg of leupeptin per ml and digested at 37°C for 15 min with a mixture of pancreatic RNase A (2 μg/ml) and 10 U of RQ DNase (Promega Biotec). The digestion was stopped by the addition of an equal volume of a buffer containing 50 mM Tris-HCl, pH 7.4, 4% sodium dodecyl sulfate (SDS), 12% (wt/vol) glycerol, and 5% (vol/vol) 2-mercaptoethanol which was heated at 95°C for 5 min. Aliquots were electrophoresed on 12.5% polyacrylamide gels in SDS. The proteins were blotted to 0.2-μm-pore-size Immobilon filters (Millipore Corp.) and reacted first with rabbit polyclonal anti-Rev antiserum or mouse monoclonal antibodies against Rev and then with the appropriate second antibody tagged with horseradish peroxidase. The immunoreactive bands were then visualized by a commercial chemiluminescence protocol (Amersham Corp.) and quantified by scanning.

Immunoprecipitation. HeLa or Cos-7 cells were routinely labeled 48 h posttransfection. For labeling, a one-third equivalent of cells from each confluent 60-mm-diameter dish was starved for 15 min in RPMI medium lacking methionine and cysteine and containing 2.5% dialyzed fetal bovine serum. The cells were then metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (2 mCi/ml) in a total volume of 0.1 ml for 1 h at 37°C. The cells were harvested and lysed as described above for immunoblotting, except that RNase and DNase treatments were in a total volume of 0.2 ml. Following the digestion, the samples were diluted with 5 volumes of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.25% Triton X-100, 0.25% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, 0.15% bovine serum albumin, 60 mM NaCl, 100 μM bound phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, and 1 μg of leupeptin per ml. The lysates were then precleared by being bound at 4°C for 1 h to *Staphylococcus* protein A beads that had been preloaded with nonimmune immunoglobulin G. The supernatants from this step were then bound to protein A beads coated with rabbit polyclonal or mouse monoclonal anti-Rev antibodies for 2 h at 4°C. The protein A beads were collected by centrifugation, rinsed, and processed for electrophoresis through 10% acrylamide gels in SDS.

Immunofluorescence microscopy. Following electroporation, 2×10^4 cells were plated on 7-mm-diameter coverslips in 24-well plates. At 48 to 72 h posttransfection, the monolayers were rinsed twice with PBS and fixed for 15 min in 4% paraformaldehyde in PBS. The cells were rinsed three times with PBS and permeabilized with 1% Triton X-100 in PBS for 3 min. They were rinsed four times in PBS and incubated with either rabbit polyclonal anti-Rev antiserum (1:200 dilution) or anti-Rev mouse monoclonal antibody (1:500 dilution) in 0.2% bovine serum albumin in PBS for 2 to 4 h. After being washed extensively, the monolayers were reacted for 1 h with fluorescein-labeled goat anti-rabbit Fab fragment (1:500) or donkey anti-mouse antiserum (1:200) in 0.2% bovine serum albumin in PBS. After several washes in PBS, the coverslips were rinsed in deionized water and mounted in MOWIOL (Calbiochem Corp.) in 15% glycerol supplemented with 5% (wt/vol) DABCO (Sigma Chemical Co.) to prevent fading. The cells were examined by regular interference or Nomarski optics and by dark-field immunofluorescence with a Zeiss Axiophot microscope.

RESULTS

Rev/MS2 coat protein fusion activates HIV-1 *gag* expression from a hybrid RNA lacking the Rev-responsive domain. To investigate whether the RRE can be replaced by another RNA, the RRE sequence in the *gag* expression vector pRMK4RRE (23) was mutated to exchange the 64-nucleotide Rev-responsive domain for a modified MS2 phage translation operator sequence of 19 nucleotides (Fig. 1, RREZ-MS). The MS2 operator had been modified to introduce two G-C base pairs to stabilize the stem region without overtly affecting the MS2 coat protein binding (6). To direct the binding of Rev to RREZ-MS RNA, MS2 coat protein was appended to the C-terminal end of the Rev. A single-nucleotide deletion at the seventh codon of the MS-C ORF, which introduced a frameshift and early termination of the coat protein (Rev/MS-D), served as a negative control. Another mutant that deleted 48 residues at the N terminus of

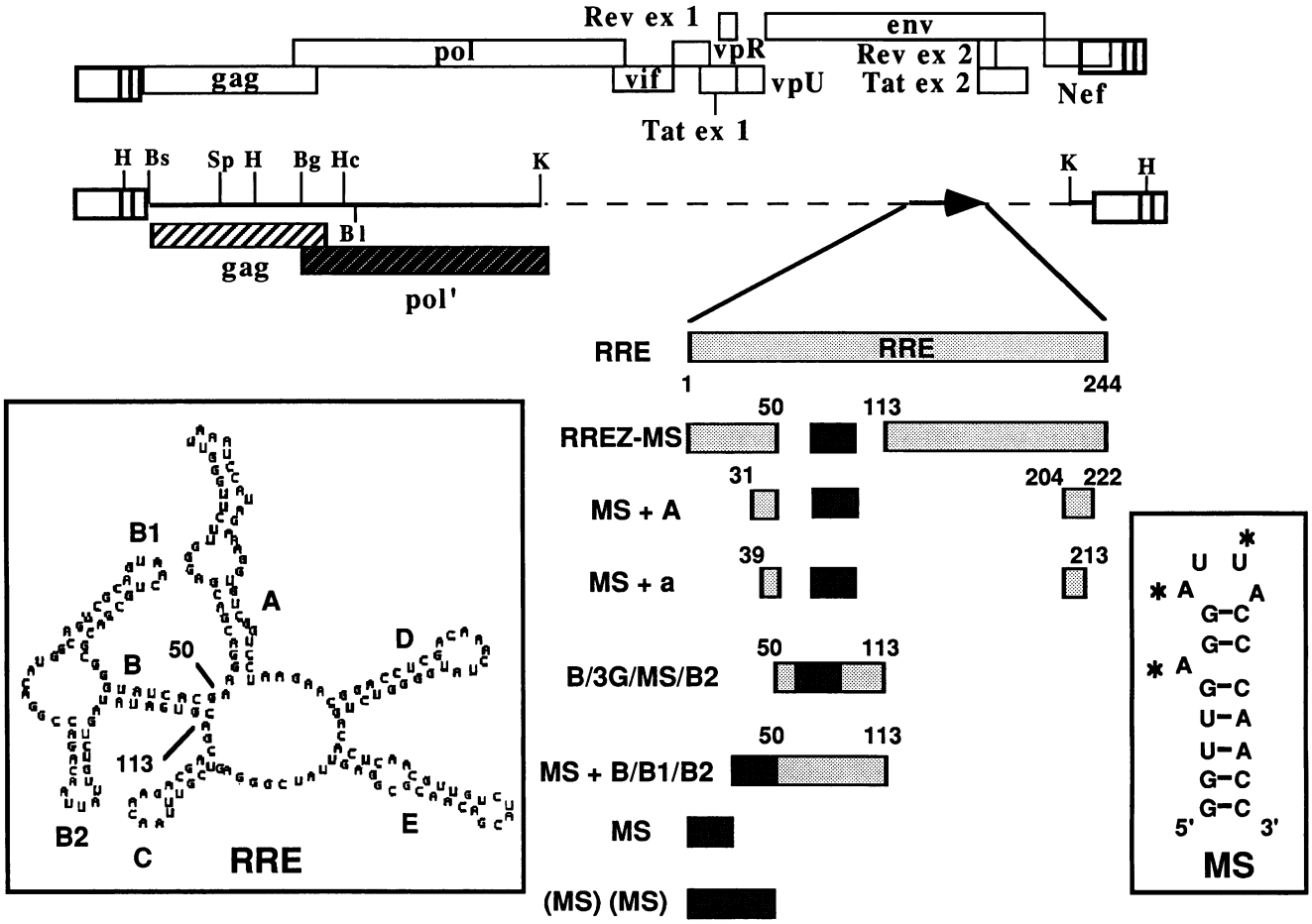


FIG. 1. Schematic diagram of HIV-1 LTR-linked *gag* expression plasmids containing RRE and RRE-MS chimeric inserts. The basic structure of the pHIVLTR-*gag*-RRE plasmid (previously referred to as pRMK4RRE) has been described before (23) and is drawn to scale under the linear genomic map of the HIV-1 provirus NL4-3 (1). The *pol* ORF in the expression plasmid was truncated at the *Kpn*I (Asp-718) site and is denoted by pol'. The letter codes refer to a few of the relevant restriction sites. The 244-nucleotide RRE sequence in this vector, indicated by the lightly shaded rectangle, had been inserted between the *Kpn*I (Asp-718) site in the *pol* ORF and the *Kpn*I site in the *nef* ORF. The predicted secondary structure of the RRE is shown in the box at left, and the individual stem-loops are labeled. The MS sequence is denoted by a heavily shaded block with the modified MS RNA sequence (6) illustrated as a stem-loop structure in the box at right. The asterisks on the MS RNA structure refer to the nucleotides critical for MS2 phage coat protein binding. The MS monomer and dimer are denoted by single and dual heavily shaded boxes. The structures of the different chimeras are indicated by combinations of lightly and heavily shaded boxes. The numbers by these boxes refer to the RRE coordinates. These chimeras are more fully described in Materials and Methods.

Rev necessary for nuclear-nucleolar targeting, RNA binding, and oligomerization was also constructed (Δ3-50Rev/MS-C). To ensure the nuclear targeting of Δ3-50Rev/MS-C, a -GRK KRR- sequence, corresponding to the nuclear localization signal of Tat protein, was inserted at the third position in the MS-C ORF of the above deletion mutant to generate Δ3-50Rev/MS-N (Fig. 3).

HeLa cells were transiently cotransfected with Rev or the respective fusion protein expression plasmids and the indicated *gag* expression vector. Aliquots of transfected cells were taken for metabolic labeling, and the labeled extracts were immunoprecipitated with anti-Rev antiserum. Alternatively, total cellular extracts were electrophoresed through SDS-polyacrylamide gels, and the fusion proteins were detected by immunoblotting. As shown in Fig. 2A and B, fusion proteins of the predicted sizes were easily detected by immunoblotting or immunoprecipitation with the anti-Rev antiserum. The subcellular distribution of the various fusion proteins was investigated by indirect immunofluorescence of fixed, permeabilized cells with anti-Rev antiserum. As expected, Rev was concentrated in the nucleoli of transfected cells (Fig. 2C). Although the Rev/MS-C fusion protein appeared concentrated in the nuclei, the protein was also uniformly expressed throughout the cytoplasm and the nuclei of many transfectants. Δ3-50Rev/MS-C, which lacked a nuclear homing signal, accumulated exclusively in the cytoplasm. Δ3-50Rev/MS-N, with the Tat nuclear targeting signal at the third codon of MS-C, had a diffuse nuclear accumulation with some hint of condensation, as well as diffuse cytoplasmic staining (Fig. 2C).

gag expression from plasmids containing the wt RRE was compared with that from plasmids containing RREZ-MS following transfection of HeLa or Cos-7 cells. In the *gag* ELISA, all of the cell extracts were serially diluted to obtain optical density values of between 1 and 2 for RRE activation by Rev. Under these conditions, the ELISA values for a positive Rev response varied between 100 and 500 times that of *gag* expression in the absence of Rev. The cutoff value

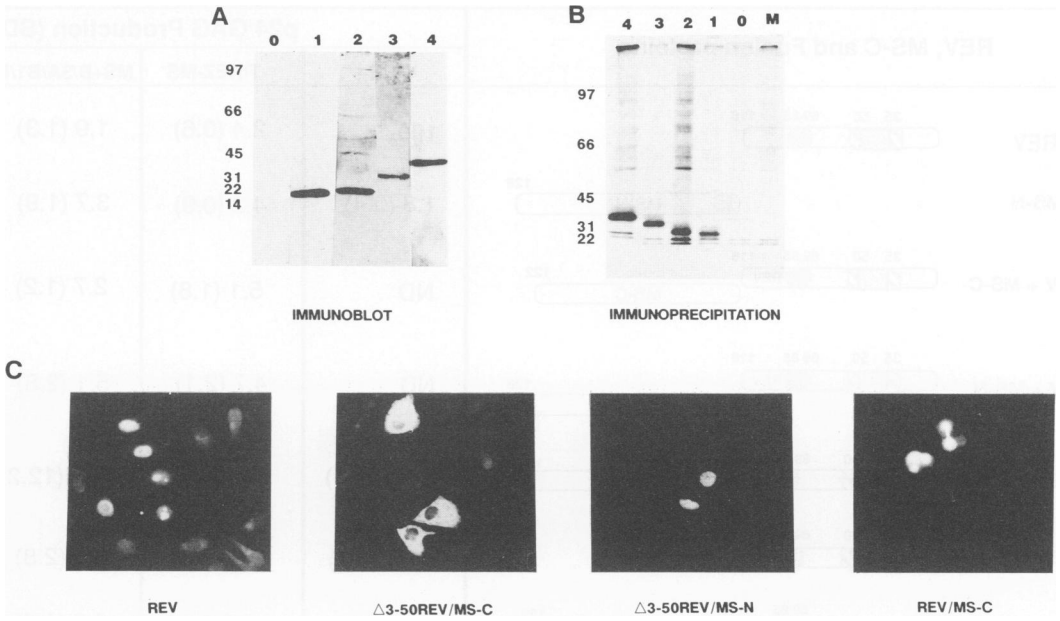


FIG. 2. Eukaryotic expression and subcellular localization of Rev and selected Rev/MS fusion proteins. HeLa cells were transfected with the respective plasmids in the context of transient *gag* expression from an HIV-1 LTR-linked, RREZ-MS-containing *gag* expression plasmid. (A) Immunoblotting. After electroporation, HeLa cells were plated on 60-mm-diameter dishes and harvested 48 to 72 h later. A one-third equivalent of cells from each dish was used for immunoblotting with polyclonal rabbit anti-Rev antiserum as described in Materials and Methods. The results from immunoblotting of 12.5% polyacrylamide gels are illustrated. Lanes: 0, no transactivator; 1, Rev; 2, Rev/MS-D; 3, Δ3-50Rev/MS-N; 4, Rev/MS-C. Molecular sizes (in kilodaltons) are indicated at the left. (B) Immunoprecipitation. Total cell lysates were prepared and processed for radioimmunoprecipitation with polyclonal rabbit anti-Rev antiserum. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis with 10% gels, and the radioactive proteins were visualized by fluorography. Lanes: M, mock transfection; 0, transfection without Rev or Rev/MS expression plasmids; 1, Rev; 2, Δ3-50Rev/MS-C; 3, Δ3-50Rev/MS-N; 4, Rev/MS-C. Molecular sizes (in kilodaltons) are indicated at the left. (C) Indirect immunofluorescence assay. After DNA was electroporated, approximately 2×10^4 HeLa cells were plated on coverslips in 12- or 24-well plates and harvested 48 to 72 h later for immunofluorescence assay. The results obtained with four different proteins are illustrated.

(recommended by the manufacturer) for the negative response was at ELISA readings that fell below 10% of RRE-Rev values after dilution. Values that ranged between 10 to 20% of those with the RRE and Rev were scored as marginal responders. *gag* expression from the RREZ-MS plasmid was induced by the Rev/MS-C fusion protein but not by Rev or the MS-C protein, alone or in combination (Fig. 3). The Rev/MS-C fusion was equally efficacious with both the RRE and the RREZ-MS plasmids (Fig. 3, row 5). When the MS ORF in Rev/MS-C was interrupted by a single-base deletion, causing a frameshift, the resulting fusion protein, Rev/MS-D, lost all activity with the RREZ-MS chimera while it preserved the activation of the RRE plasmid (Fig. 3, row 6). The deletion of 48 residues near the N terminus of Rev generated a fusion protein (Δ3-50Rev/MS-C) that was predominantly localized to the cytoplasm and was inert with both the RRE and the RREZ-MS targets (Fig. 3, row 7). Insertion of the Tat nuclear targeting signal into Δ3-50Rev/MS-C allowed the resulting mutant, Δ3-50Rev/MS-N, to accumulate in the nucleus but did not restore the activation for the RRE. Δ3-50Rev/MS-N was marginally active with the RREZ-MS target (Fig. 3, row 8), which suggested that although Δ3-50Rev/MS-N most likely bound RREZ-MS via the MS2 coat protein end, the N terminus of Rev was required for the activation of the heterologous target.

MS operator mutants with reduced in vitro coat protein affinities have corresponding decreases in the in vivo Rev response. To examine whether *trans* activation of the RREZ-MS chimera by the fusion protein was dictated by the binding of the MS coat protein, the MS2 operator was mutagenized to introduce changes known to reduce coat protein binding in vitro. Deletion of the entire MS sequence (RREZ) resulted in the loss of the Rev response (Table 1). Removal of the single bulged A residue in the stem structure of the MS2 operator has been shown to reduce the in vitro binding affinity of the coat protein by greater than 2 orders of magnitude (10, 40). An RREZ-MS chimera with this deletion (RREZ-MSΔA) was defective for *trans* activation by Rev/MS-C (Table 1). Changes at two other positions in the -AUUA- loop sequence [RREZ-MS(U/A) and RREZ-MS(A/C)] known to diminish in vitro binding by at least 10-fold (10, 40) led to a moderate decrease or no change in the in vivo response to the fusion protein (Table 1).

To demonstrate directly that the Rev/MS-C fusion retained the authentic binding properties of the Rev and MS coat proteins, the Rev/MS-C protein was overexpressed in *E. coli* as a fusion protein tagged to the C-terminal end of the MBP in the sense (MBP/Rev/MS-C) or the antisense (MBP/Rev/MS-C) orientation. Additionally, a derivative of Rev/MS-C carrying a defective interfering mutation in the activation domain of Rev [MBP/Rev(LE/PE)/MS-C] was also expressed and purified. The MBP fusion proteins were induced with IPTG and purified from inclusion bodies by affinity chromatography on amylose resin. A single major band of ca. 68 kDa was visualized in the affinity-purified fractions from the MBP/Rev/MS-C and the MBP/Rev(LE/PE)/MS-C recombinants (Fig. 4A). The (MBP/Rev/MS-C) recombinant synthesized an elongated MBP tagged at the C

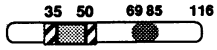
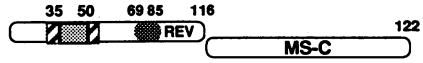
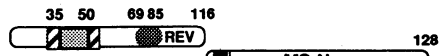
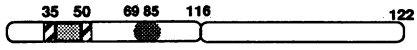
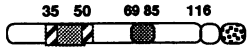
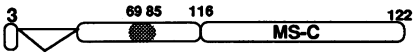
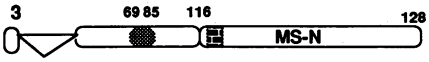
	REV, MS-C and Fusion proteins	p24 GAG Production (SD)			
		RRE	RREZ-MS	MS+B/3A/B1/B2	(MS) (MS)
1		100	2.4 (0.6)	1.9 (1.3)	6.8 (2.6)
2		1.8 (0.4)	4.2 (0.9)	3.7 (1.9)	2.8 (1.4)
3		ND	5.1 (1.8)	2.7 (1.2)	8.2 (2.3)
4		ND	4.7 (2.1)	5.1 (2.8)	6.7 (2.7)
5		**111 (17.8)**	**143 (19.7)**	**113 (12.2)**	**42.7 (8.7)**
6		**54.3 (9.3)**	5.7 (2.8)	6.1 (2.8)	4.3 (1.3)
7		4.1 (2.3)	1.7 (0.8)	3.2 (1.8)	5.7 (2.3)
8		6.3 (2.8)	19.3 (4.1)	27.7 (5.8)	9.1 (3.8)

FIG. 3. *trans* activation of HIV-1 *gag* mRNAs containing RRE, RREZ/MS, (MS)+B/3A/MS/B2, and [(MS)(MS)] targets to Rev and selected Rev/MS fusion proteins under transient conditions in HeLa cells. The ORFs of the various transactivators are schematically illustrated. In each case, the shaded square near the N terminus of Rev represents the Arg-rich domain required for RNA binding and nuclear-nucleolar localization, and the rounded, shaded rectangle corresponds to the leucine-rich activation domain. The hatched regions flanking the RNA binding motif represent the putative oligomerization domains. In MS-N, six amino acids from the nuclear localization domain of Tat have been inserted at the third position of MS-C (6). MS-D refers to a single-nucleotide deletion at the sixth codon of MS-C which results in a frameshift and early termination (6). *gag* expression values, normalized for transfection efficiency, are tabulated with the respective standard deviations (SD) derived from at least eight independent experiments. The positive values are denoted by bold typeface, and intermediate values are underlined. Rev-dependent *gag* expression from a wt RRE-containing plasmid is arbitrarily assigned a value of 100. The results of this and all other subsequent experiments were also duplicated with Cos-7 cells. ND, not done.

terminus by 12 residues encoded by the antisense strand of the Rev/MS-C gene. The major 68-kDa band in the lysates from the MBP/Rev/MS-C and MBP/Rev(LE/PE)/MS-C cells immunoreacted with murine monoclonal anti-Rev antibody (Fig. 4B). The faster-migrating immunoreactive bands in Fig. 4B probably represent a protein breakdown product(s). When the purified MBP fusion proteins were incubated with [α^{32}P]UMP-labeled RRE or RREZ-MS RNA, ribonucleoprotein (RNP) complexes were formed only with the MBP/Rev/MS-C protein and not with the fusion containing the antisense Rev/MS-C (Fig. 4C). Under the same conditions, MBP/Rev(LE/PE)/MS-C fusion protein bound both RRE and RREZ-MS RNAs, while purified Rev bound only RRE RNA (data not shown). When purified MBP/Rev/MS-C protein was incubated with RREZ, RREZ-MS, or RREZ-MSΔA RNAs, RNP complexes were formed only with the RREZ-MS RNA and not with the RREZ or the RREZ-MSΔA chimera with a deleted bulged A residue (Fig. 4D).

TABLE 1. In vivo responses of mutations in the MS sequence of RREZ-MS to Rev and Rev/MS-C

RRE or RRE-MS RNA chimera[a]	p24^{gag} production (± SD) relative to RRE and Rev in the presence of:		
	No transactivator	Rev	Rev/MS-C
wt RRE	2.2 (1.5)	100	111 (17.8)
ERR (reverse RRE)	1.8 (1.1)	3 (1.3)	2 (1.4)
RREZ	1.8 (1.4)	3.1 (1.7)	6.3 (2.7)
RREZ-MS	4.8 (2.4)	2.8 (1.4)	143 (19.7)
RREZ-MSΔA	3.7 (1.8)	3.7 (1.8)	12.3 (6.8)
RREZ-MS(U/A)	7.8 (3.2)	1.8 (0.9)	49 (7.8)
RREZ-MS(A/C)	10.7 (4.3)	4.7 (1.3)	87 (14.6)

[a] In addition to the deletion of the entire MS sequence (RREZ), three point mutations were introduced in the MS stem-loop sequence in the context of the RREZ-MS target: (i) deletion of the single bulged A in the MS stem (RREZ-MSΔA), (ii) substitution of A for U in the -AUUA- loop sequence [RREZ-MS(U/A)], and (iii) substitution of C for A in the -AUUA- loop sequence [RREZ-MS(A/C)]. Normalized GAG-expression values are shown.

Definition of the minimal RRE/MS chimera that is responsive to Rev/MS-C fusion protein in vivo. To examine whether the RRE sequences in the RREZ-MS target contribute to activation by Rev/MS-C fusion protein (by recruiting cellular factors, etc.), the stem-loops C, D, and E and the central loop of the RRE were excised from RREZ-MS to generate (MS)+A and (MS)+a. In (MS)+A, RRE stem A sequences corresponding to positions 31 to 50 and positions 204 to 222 were placed 5′ and 3′, respectively, of the MS sequence. RRE sequences between positions 39 and 50 and between

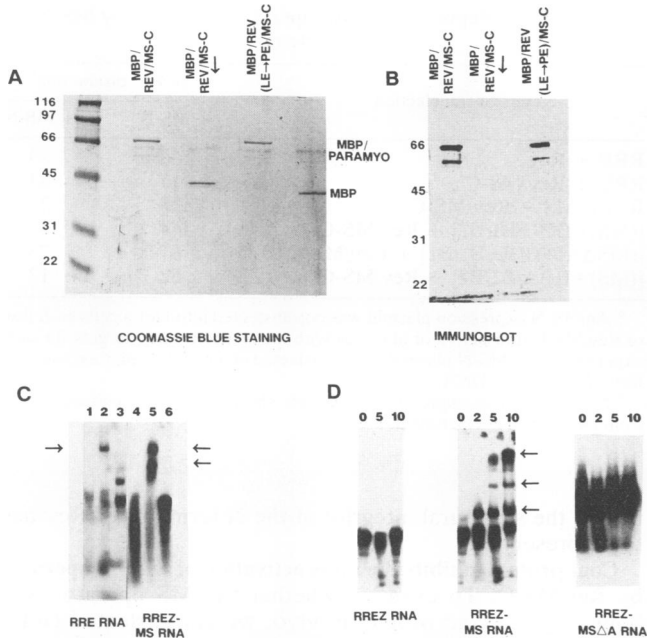


FIG. 4. Characterization of Rev/MS fusion proteins expressed in *E. coli*. Rev/MS-C and Rev(LE/PE)/MS-C proteins were expressed in *E. coli* as MBP fusion proteins and purified as described in Materials and Methods. (A) Coomassie blue staining of the respective fusion proteins purified by affinity chromatography and resolved by SDS-polyacrylamide gel electrophoresis with 12% gels. Results obtained with Rev/MS-C fused to MBP in the sense and antisense (underlined by arrow) orientations and MBP/Rev(LE/PE)/MS-C fusion are shown. LE→PE refers to the substitution of leucine for proline in the -LE- sequence in the activation domain of Rev. Molecular mass markers were run on the left lane, and sizes (in kilodaltons) are indicated. Purified MBP and paramyosin-MBP fusion proteins were obtained commercially and electrophoresed as indicated on the right. (B) Immunoblot of the respective fusion proteins. Inclusion bodies from *E. coli* expressing the various fusion proteins were solubilized and electrophoresed on 12% gels in SDS. The proteins were electroblotted and reacted with murine anti-Rev monoclonal antibodies. Immunoreactive bands were visualized by a commercial chemiluminescence protocol (Amersham Corp.). The faster-migrating band probably represents a breakdown product(s). (C) Gel mobility shift analysis of RNA binding by Rev/MS-C fusion proteins. The affinity-purified fusion proteins described for panel A were incubated with [α-^{32}P]UMP-labeled RRE RNA or RREZ-MS RNA, and the formation of RNA-protein complexes (RNPs) was evaluated by polyacrylamide gel electrophoresis as described in Materials and Methods. Lanes: 1 and 4, control lanes without *E. coli* proteins; 2 and 5, results obtained with MBP/Rev/MS-C fusion protein; 3 and 6, MBP ORF fused to the antisense strand of Rev/MS-C. The arrows denote the RNPs. The slow-migrating band in lane 3 represents a conformational isomer of the RRE and not the RNP. (D) Sequence-specific binding by MBP/Rev/MS-C fusion protein. Increasing concentrations (top) of affinity-purified MBP/Rev/MS-C protein were incubated with constant molar equivalents of [α-^{32}P]UMP-labeled RREZ, RREZ-MS, and RREZ-MSΔA RNAs, and the RNP complexes were analyzed by polyacrylamide gel electrophoresis.

positions 204 and 213 flanked the MS sequence in (MS)+a (Fig. 1). Both (MS)+A and (MS)+a were *trans*-activated at levels somewhat lower than the RREZ-MS activation (Table 2). As expected, neither chimera responded to Rev alone. This led us to pursue whether MS sequence alone may be sufficient to target the fusion protein in vivo. A recombinant

TABLE 2. Minimal heterologous RNA recognized by Rev/MS-C in vivo

RRE or RRE-MS RNA chimera[a]	p24^{gag} production (± SD) relative to RRE and Rev in the presence of[b]:		
	No transactivator	Rev	Rev/MS-C
wt RRE	2.0 (0.4)	100	111 (17.8)
ERR (reverse RRE)	1 (0.7)	3 (1.3)	2 (1.4)
RREZ-MS	2.3 (0.9)	2.8 (1.4)	143 (19.7)
(MS)+A	2.8 (1.5)	1.8 (0.6)	32.8 (6.8)
(MS)+a	0.8 (0.5)	0.8 (0.6)	45.8 (9.8)
(MS)	4.8 (1.9)	3.7 (1.3)	17.1 (6.2)
(SM)	5.1 (1.3)	ND[c]	4.1 (2.2)
MSΔA)	6.1 (4.4)	3.7 (1.3)	3.1 (1.2)
(MS)(MS)	9.1 (5.4)	3.9 (2.2)	42.7 (8.7)
(MS)(SM)	8.4 (4.7)	ND	29.7 (9.2)

[a] Three types of minimal targets were analyzed: (i) MS sequence flanked by the 5′ and 3′ strands of stem A of RRE [(MS)+A and (MS)+a in Fig. 1], (ii) complete exchange of the RRE with the MS sequence in the sense (MS) or antisense (SM) orientation or with an MS mutant which deleted the single bulged A residue (MSΔA), and (iii) a head-to-tail [(MS)(MS)] or head-to-head [(MS)(SM)] dimer of the MS sequence.

[b] *gag* expression from the respective targets in response to Rev or Rev/MS-C or that in their absence is tabulated as described in the legend to Fig. 3.

[c] ND, not done.

that completely exchanged the RRE for the MS sequence (MS) was poorly activated by Rev/MS-C, while the MS sequence in reverse orientation (SM) and the MS sequence with the deleted A bulge (MSΔA) were not responsive to Rev or Rev/MS-C (Table 2). However, when a head-to-tail dimer [(MS)(MS)] linked by a 6-nucleotide palindromic Asp-718 site (-GGTACC-) was inserted in place of the RRE, the resulting RNA had a modest response compared with that of RREZ-MS (Table 2). A head-to-head dimer [(MS)(SM)] was less efficient for the Rev/MS-C-dependent response (Table 2). These results imply that the MS operator sequence alone is sufficient to tether the fusion protein if the operator structure is exposed for protein binding.

In vivo responses of the bivalent RNAs correlate with their predicted binding affinities for Rev and MS-C. Rev/MS-C *trans*-activated both RRE and RREZ-MS RNA, presumably by its ability to bind the RRE by the N-terminal end of Rev and the MS operator by the coat protein moiety. An RNA molecule containing both of these targets may be inactivated at one or both of the sites of protein binding and used to uncouple the binding and the activation events for the fusion protein. Two bivalent chimeras were designed: (i) B/3G/MS/B2, which replaced the B1 stem-loop in the Rev-responsive B/3G/B1/B2 RRE subdomain with the operator sequence but left the critical three-G sequence intact, and (ii) (MS)+B/B1/B2, with tandemly placed MS and B/B1/B2 sequence elements (Fig. 1). Neither of these plasmids expressed *gag* in the absence of Rev or Rev/MS-C. B/3G/MS/B2 was activated by Rev/MS-C at 40% of the levels observed with RREZ-MS (Table 3). In the presence of Rev, *gag* expression from B/3G/MS/B2 was about 24% of that from an equivalent RRE plasmid (Table 3). A recombinant that had tandemly placed MS and B/B1/B2 targets [(MS)+B/B1/B2] was activated by both Rev and Rev/MS-C to levels exceeding those obtained with the RRE and Rev or RRE/MS-C and Rev/MS-C, respectively (Table 3).

The above bivalent chimeras were mutated to restrict their targeting for the Rev or MS-C component of the fusion protein. When the three G's in the Rev-responsive subdomain were replaced by three A's, Rev was no longer able to

TABLE 3. In vivo Rev responses of wt and mutant bivalent chimeras

RRE or RRE-MS RNA chimeras[a]	$p24^{gag}$ production (± SD) relative to RRE and Rev in the presence of[b]:		
	No transactivator	Rev	Rev/MS-C
wt RRE	2.0 (0.4)	100	111 (17.8)
RREZ-MS	2.3 (0.9)	2.8 (1.4)	143 (19.7)
B/3G/MS/B2	7.8 (3.8)	24 (6.5)	59.7 (11.3)
B/3A/MS/B2	7.8 (3.8)	5.1 (2.5)	43.7 (9.3)
B/3G/MSΔA/B2	5.2 (2.2)	22 (8.2)	37.7 (8.3)
(MS)+B/3G/B1/B2	4.3 (1.2)	137 (21.8)	173 (18.4)
(MS)+B/3A/B1/B2	5.5 (3.2)	7.2 (1.8)	94.3 (12.4)
(MSΔA)+B/3G/B1/B2	3.8 (1.8)	96.7 (9.8)	87.3 (6.7)
(MSΔA)+B/3A/B1/B2	6.3 (2.8)	2.7 (0.8)	9.3 (4.9)

[a] Two plasmids containing targets for both Rev and the coat protein were used. In B/3G/MS/B2, the MS sequence replaced the B1 stem-loop in the Rev-responsive RRE subdomain B/B1/B2 but left the three G's of the RRE intact. In (MS)+B/B1/B2, the MS sequence was linked to the downstream Rev-responsive B/B1/B2 subdomain of the RRE by three A's. The chimeras were mutagenized to replace the three G's in the Rev-responsive subdomain with three A's, to delete the single bulged A in the MS operator, or to do both.

[b] *gag* expression values in the presence of Rev or Rev/MS-C or those in their absence are shown.

TABLE 4. Repression of MS operator activation by MS-N coat protein[a]

Type of transfection	$p24^{gag}$ production[b]	
	− MS-N	+ MS-N
RRE + Rev	100	89
RRE + Rev/MS-C	117	84
RREZ-MS + Rev/MS-C	135	23
[(MS)+B/3G/B1/B2] + Rev/MS-C	144	87
[(MSΔA)+B/3G/B1/B2] + Rev/MS-C	87	73
[(MS)+B/3A/B1/B2] + Rev/MS-C	82	17

[a] An MS-N expression plasmid was cotransfected into HeLa cells with Rev or Rev/MS-C transactivator plasmids with the respective *gag* targets. In each experiment, the MS-N plasmid was transfected at a sixfold molar excess over Rev or Rev/MS-C DNA.

[b] Normalized *gag* expression values are shown. Data are average values from two or three experiments.

activate the resulting chimera (Table 3). The change of three G's to three A's in the context of the full RRE has previously been demonstrated to lose the Rev response without overtly affecting the folding dynamics of the RRE structure (23, 24). The three-G-to-three-A transition mutant resulted only in a slight to moderate diminution in the activation response to Rev/MS-C (Table 3), since these mutants retain the binding potential for MS-C. In contrast, a mutation which deletes the bulged A residue may lose coat protein binding but can still be bound by Rev. Accordingly, deletion of the bulged A in MS induced only a modest diminution of the in vivo response to both Rev and Rev/MS-C (Table 3). Mutating the (MS)+B/B1/B2 RNA at both the three G's and the A bulge in the MS operator caused a severe impairment of activation by Rev/MS-C and a complete loss of the Rev response (Table 3).

A comparison of the in vivo responses of [(MS)(MS)], (MS)+B/3A/MS/B2, and RREZ-MS to Rev and combinations of the Rev and Rev/MS fusion proteins is shown in Fig. 3. All three chimeras lack Rev recognition sites and are exclusively activated by the Rev/MS-C fusion protein (Fig. 3, row 5) but not by Rev, MS-C, or MS-N, alone or in combination (Fig. 3, rows 1 to 4). All three univalent targets also failed to respond to the Rev/MS-D fusion protein containing a prematurely terminated MS coat protein (Fig. 3, row 6). The differential responses of the three chimeras to Rev/MS-C probably reflect the intrinsic stability of the MS RNA secondary structure in the various molecules rather than the requirements for additional sequence to facilitate protein binding. Therefore, if the RNA can bind the fusion protein, Rev moiety will activate that RNA. A large deletion at the N terminus of Rev in the fusion protein (Δ3-50Rev/MS-C) abolished *trans* activation (Fig. 3, row 7), most likely because of the loss of nuclear targeting (Fig. 2C). Insertion of the HIV-1 Tat nuclear homing signal in the coat protein moiety of the above deletion mutant generated Δ3-50Rev/MS-N, which was distributed in both the nuclear and cytoplasmic compartments (Fig. 2C). However, with the Δ3-50Rev/MS-N protein, only a fraction of the *trans*-activation potential of Rev/MS-C was realized (Fig. 3, row 8), implying that for optimal *trans* activation of even a heterologous RNA target, the structural integrity of the N terminus of Rev has to be preserved.

Coat protein inhibits the *trans* activation of the MS operator by Rev/MS-C. To examine whether the MS operator was bound by the coat protein in vivo, we cotransfected HeLa cells with the MS-N expression plasmid under conditions of *trans* activation by Rev or Rev/MS-C. MS-N protein contained the HIV-1 Tat nuclear localization signals and was distributed in both the nuclear and the cytoplasmic compartments. MS-N would be expected to complete directly with the fusion protein for the targets containing the MS operator but not for the Rev-responsive target. As shown in Table 4, a sixfold molar excess of MS-N over Rev/MS-C induced a sevenfold decrease in the *trans* activation of RREZ-MS by the Rev/MS-C fusion protein; activation of the RRE either by Rev or by the fusion protein was not significantly affected. Activation of the bivalent RNA and the mutant chimera restricted to Rev binding was also not significantly influenced by MS-N coexpression (Table 4). In contrast, MS-N caused a fivefold reduction in Rev/MS-C-dependent *gag* expression from a chimera containing the three-A mutation in the Rev-responsive motif (Table 4).

Activation by the fusion protein requires the Rev activation domain. Genetic studies have identified an Leu-rich motif near the C terminus of Rev as the activation domain. Mutations in this domain were not only inactive for *trans* activation but also dominantly interfered with the functioning of wt Rev (31, 34). To examine whether *trans* activation of the heterologous RNA by the fusion protein was dependent on the integrity of the activation domain of Rev, we designed two substitution mutants with changes in the Leu-rich motif of Rev in the context of the fusion protein. Both Rev(78-79LE/EE)/MS-C and Rev(78-79LE/PE)/MS-C proteins were inactive for *gag* expression from RRE plasmids; they were marginally active (10 to 20% of the levels obtained with Rev/MS-C) on the RREZ/MS-C plasmid (data not shown). However, when increasing amounts of either mutant were cotransfected with the wt Rev/MS-C, there was a dose-dependent repression of *trans* activation (Fig. 5). Under similar conditions, cotransfection of a molar excess of Δ3-50Rev/MS-C was without an inhibitory effect (data not shown). The mutant (-LE- to -PP-) was more inhibitory than the change (-LE- to -EE-). *trans* activation of the RRE or (MS)+B/3A/B1/B2 containing *gag* plasmids by the wt fusion protein was repressed more efficiently (90% inhibition at a fivefold molar excess) by the same Leu motif mutants (data not shown). These results, which parallel the previously

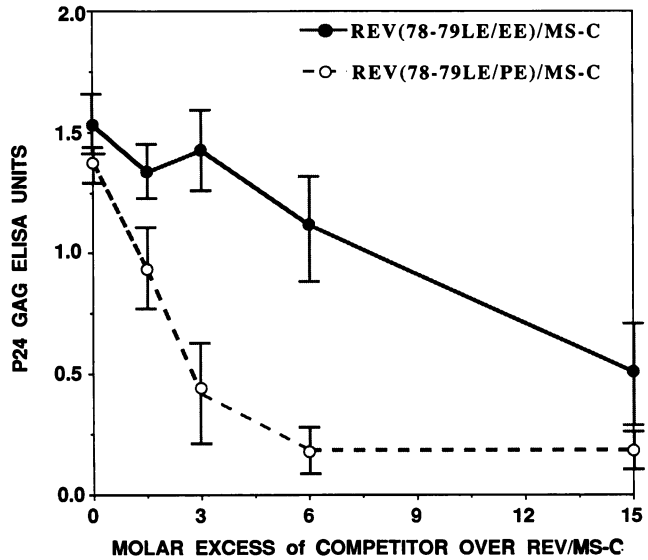


FIG. 5. Effects of dominant interfering mutations near the C terminus of Rev on *trans* activation by Rev/MS-C. Increasing amounts of RSV LTR-linked Rev/MS-C proteins with substitutions at the 78 and 79 residues of the Rev ORF were cotransfected with fixed amounts of Rev/MS-C and RREZ-MS *gag* plasmid. Normalized values of $p24^{gag}$ production are presented as a function of the competitor concentration. Conditions are as described for other experiments.

reported behavior of activation domain mutants of Rev on the RRE (31, 34), imply common biochemical pathways of activation for both Rev and Rev/MS-C fusion proteins.

DISCUSSION

We have demonstrated that direct interaction of Rev with the RRE RNA is not an absolute requirement for *trans* activation. Rev will activate a heterologous RNA if it is directed to it by another RNA binding protein. When the Rev-binding domain of the RRE was replaced by the MS2 phage operator sequence (RREZ-MS), Rev/MS coat protein fusion activated the respective *gag* mRNA at levels slightly greater than those for the corresponding activation of the RRE by Rev. Mutations in the MS operator known to abolish or reduce in vitro binding by the coat protein demonstrated a corresponding loss of in vivo response to the fusion protein. This point was directly confirmed by the in vitro binding preferences of the purified fusion protein with the various RNAs. The purified fusion protein bound the RRE and RREZ-MS RNAs but not the RREZ or RREZ-MSΔA RNAs.

Although the RREZ-MS chimera was fully functional for the Rev response, RNA with a complete exchange of the RRE with the MS sequence was activated by the fusion protein to less than 20% that of RRE activation by Rev. Activation of the MS stem was slightly improved when it was extended to include the sequence corresponding to the RRE stem A. This improvement was not attributable to the sequence content of the RRE, since similar enhancement was also observed by lengthening the MS stem by an unrelated sequence (data not shown). A head-to-tail or a head-to-head MS dimer also showed a modest increase in response over the MS monomer response to the fusion protein. A deletion mutant of the RRE (RREZ) that removed the Rev-responsive domain (B/B1/B2) was activated neither by Rev nor by the fusion protein. Therefore, the MS sequence alone is sufficient to tether the fusion protein during *trans* activation if this sequence forms a stable secondary structure in the context of the rather large HIV-1 *gag-pol* mRNA, as in the cases of RREZ-MS and, to a lesser degree, the other minimal MS chimeras.

The fusion protein was equally effective against both the RRE and the RREZ-MS targets. Cotransfection of RRE and RREZ-MS expression plasmids resulted in an additive response when the fusion protein was not limiting (data not shown). Direct proof for the dual-RNA attachment potential of the fusion protein was obtained with a bivalent chimera that contained the MS operator and the RRE subdomain in tandem [(MS)+B/3G/B1/B2]. As summarized in Fig. 6, the bivalent target was activated by both Rev and Rev/MS-C. Substitution of the three G's by three A's in the Rev-responsive subdomain selectively abolished the response to Rev but not that to Rev/MS-C. When the coat protein sequence in Rev/MS-C was truncated, the resulting mutant, Rev/MS-D, failed to activate the MS2-only targets, emphasizing the requirement of the coat protein end for RNA binding. In vivo binding of the fusion protein to MS2 RNA was indirectly demonstrated by competition experiments with excess quantities of the coat protein, MS-N. MS-N inhibited the activation of operator-only targets by the fusion protein but had no effect on RRE activation. Thus, the RNA binding and activation properties of Rev may be structurally and functionally dissociated without the fidelity of the activation process being lost.

The in vivo reactivities of the bivalent chimeras reiterated that RNA attachment by either binding protein is highly sequence specific. Although sequence-specific interactions of MS coat protein with the MS operator have been well documented, the situation regarding RRE/Rev interactions has been equivocal. Recent reports have in fact claimed that Rev binding of the RRE may be specified not by a unique sequence in the B/B1/B2 RRE subdomain but rather by the unusual distortions in the sugar phosphate backbone imposed by noncanonical base-pairing schemes (3, 18, 28). We have demonstrated previously that Rev binding and the Rev response are preserved in mutants that exchanged the B1 stem-loop for a perfectly Watson-Crick base-paired MS2 helix loop both with Rev (24) and with Rev/MS-C fusion proteins. The loss of both the in vitro and in vivo Rev responses as a consequence of the three G's being mutated upstream of the MS sequence in these constructs argues in favor of sequence-specific binding of Rev to the B/B1 stem-loop region, both in its native context and as the Rev/MS-C fusion protein.

Several studies have implied that for optimal activation by Rev, host cellular factors binding to RRE RNA, Rev, or both may be required. Several RRE RNA binding nuclear proteins were demonstrable by a UV cross-linking procedure (36a), including the previously reported 56-kDa RBF protein (44). The case for the RRE-binding Rev helper factors is supported by the inability of certain RRE mutants that bind Rev to be *trans*-activated by it (23) and by the restricted tropism of the Rev response (43). Certain RRE mutants that bound Rev poorly and were not Rev responsive in vivo still facilitated *gag* expression in the absence of Rev, implying the presence of a cellular surrogate(s) for Rev (24). Our best non-RRE constructs were rescued by the Rev/MS-C fusion protein to only about 42% of the RRE by Rev, suggesting that RRE sequence recognition by cellular factors may also improve activation by the fusion protein. However, our

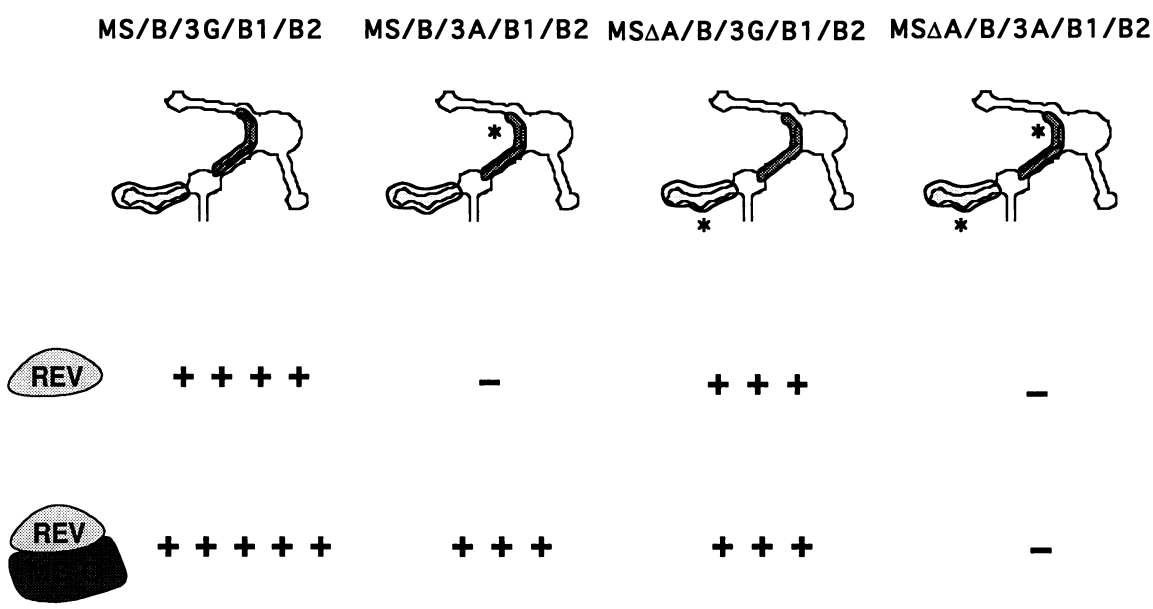


FIG. 6. Schematic representation of RNA motifs recognized by Rev and MS coat proteins. A chimera linking the MS operator sequence to the Rev-responsive B/B1/B2 subdomain of the RRE was maximally responsive to both Rev and Rev/MS-C. The cognate targets for the MS-C and Rev proteins are denoted by the lightly and heavily shaded areas, respectively. The asterisks refer to the change of three G's to three A's in the RRE subdomain and to the deletion that removed the bulged A in the MS operator. The relative Rev responses of the various mutants are shown.

substitution mutation of three G's to three A's (3G/3A) in the bivalent chimera was not activated by Rev or the Rev/MS-D mutant that truncated the coat protein. Besides the MS2 RNA, the 3G/3A mutant contained only the non-Rev-responsive subdomain of the RRE. Furthermore, a 3G/3A substitution mutant, B/3A/MS/B2, that contained an even smaller Rev-responsive sequence (B and B2 stem-loops of the B/B1/B2 subdomain) was also nonresponsive to Rev, although not to the fusion protein. While these observations underscore the fact that Rev binding to the target is the primary event during activation, it is still possible that RRE-binding cellular factors may regulate the overall efficiency of the Rev response.

The amino-terminal peptide domain(s) of Rev is necessary for RNA binding, nuclear-nucleolar localization, and oligomerization properties (8, 25, 27, 31). Δ3-50Rev/MS-C with a 48-amino-acid deletion at the N terminus of Rev accumulated preferentially in the cytoplasm and was therefore inactive with both the RRE and the MS chimeras. When the Tat nuclear localization sequence was inserted at the third codon of the MS-C ORF in the above deletion, the resulting fusion protein, Δ3-50Rev/MS-N, was distributed equally between the cytoplasm and the nucleus and possibly in the nucleolus. Δ3-50Rev/MS-N failed to activate the RRE target and was only partially active with the MS2 operator. Since Δ3-50Rev/MS-N lacks the RRE-binding site, its failure to activate the RRE is understandable. But its partial in vivo effect with the MS operator targets implies that the amino terminus of Rev may possess an additional function(s) required for maximal activation. Binding to heterologous RNA targets is probably not one of these properties, since the Rev/MS-D fusion, with an intact Rev ORF but a truncated coat protein ORF, activated only the RRE but not the MS targets. Since Δ3-50Rev/MS-N is not excluded from the nucleus, a loss of protein oligomerization potential may be the critical defect in Δ3-50Rev/MS-N. However, the MS coat protein itself can dimerize and form higher-order structures upon specific RNA binding (4, 48). It is likely that the Δ3-50Rev/MS-N fusion may have retained this property of the MS2 coat protein. Therefore, the Δ3-50Rev/MS-N deletion mutant must lack yet another function encoded by the N terminus of Rev. Even substitution mutants of Rev/MS-C that replaced one or more arginine residues in the N terminus of Rev also had a negative phenotype with the RRE and were only marginally active with the MS operator targets (45a). Assuming that the various mutations in the Rev N terminus do not drastically affect fusion protein folding, we can speculate on the possible roles of the Rev N terminus. First, even if RNA binding occurs at the coat protein moiety, the fusion protein may still have to oligomerize by using the N-terminal domain of Rev. Alternatively, activation may require cooperative intramolecular interactions between the heterologous RNA binding site and the intact N terminus of Rev or intermolecular interactions between the N termini of Rev in different fusion protein molecules. Finally, an authentic N terminus of Rev may be required for binding of cellular factors.

In the life cycle of HIV, the Rev protein regulates the temporal switch from the early regulatory phase to the late lytic phase (2, 13, 37, 41). HIV replication may be restricted at the level of Rev from constraints of the threshold requirements of Rev (33, 38, 39), by the requirement of species-specific cellular helper factors (43), or by both. Rev is presumed to be a determinant of viral latency and reactivation during natural infection (38, 39) and is therefore an excellent candidate for targeted drug development. Like many activators, Rev is a modular protein, with the N-terminal and the C-terminal halves subserving the RNA binding and the activation functions, respectively. Mutants with changes in the leucine-rich activation domain bind the RRE, but they are defective for activation and dominantly interfere with the Rev function (31), presumably by forming mixed

oligomers (26, 33). However, it is equally plausible that the activation domain interacts with specific cellular factors modulating the RNA spliceosome assembly or transnuclear RNA transport (11, 30, 31). The activation domain mutants also inhibited activation by the fusion protein, implying common biochemical pathways of activation for both Rev and Rev/MS-C fusion proteins. While the activation domain is being explored as a potential target for drug development, the fusion protein system we have developed is useful for examining the various RNAs as potential Rev decoys. Using a distant RNA binding site in the fusion protein rather than the RRE-binding domain may uncover the in vivo RNA binding parameters and also identify cross talk between the heterologous RNA binding site and the Rev oligomerization domain at the N terminus. The need for an authentic N-terminal domain of Rev for the activation of even the heterologous targets prompts us to examine the contributions of the different residues at this domain to the activation process. These studies will allow us to synthesize "designer" peptides that may interfere with the discrete steps leading to RNA binding, protein-protein interaction, and activation.

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