Perturbation of the Carboxy Terminus of HIV-1 Rev Affects Multimerization on the Rev Responsive Element

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ABSTRACT: Perturbations within the transactivation and carboxy-terminal domains of HIV-1 Rev were examined for effects on Rev responsive element (RRE) binding activities *in vitro* and biological activity *in vivo*. Binding affinities, specificities, and multimerization of the transactivation mutants M10 and Rev/Rex M10-16 on the RRE were equivalent to wild-type Rev. Substitution of the Rex transactivation domain within Rev resulted in the incorporation of an internal methionine residue which, when cleaved with CNBr and subsequently purified, produced a protein species (CNBr-Rev) unable to fully multimerize on the RRE. Instead, two discrete protein-dependent species were generated in the gel shift assay. Furthermore, CNBr-Rev was observed to bind to the RRE with high specificity and an equilibrium binding constant of 6×10^{-10} M. A C-terminal Rev deletion mutant (Rev M9Δ14) lacking amino acids 68–112 displayed identical RRE binding characteristics to the CNBr-Rev protein. This protein, which lacks both the activation and the C-terminal domains, was biologically inactive but maintained the ability to discriminate the RRE from nonspecific RNA. Deletion of amino acids 92–112 resulted in a Rev mutant (Rev M11Δ14) which bound to the RRE with wild-type affinity and high specificity. This purified mutant was observed to be aberrant in multimerization activity on the RRE with reduced multimerization apparent in the gel shift assay. However, Rev M11Δ14 possessed biological activity equivalent to wild-type Rev in a cell-based p24 ELISA assay. These results suggest that polymerization on the RRE is dispensable for Rev activity and that two monomeric Rev proteins bound to the RRE are sufficient for biological activity. Furthermore, *in vivo* experiments using the Rev/Rex chimeric mutant and the M10 transdominant mutant as well as *in vitro* dissociation rate studies with Rev M11Δ14 and Rev M9Δ14 suggest that the M9 through M11 domain of the protein may be involved in RRE-dependent specific Rev dimerization.

HIV-1 Rev is a sequence-specific RNA binding protein which possesses a tendency to self-associate *in vitro* (Daly et al., 1989; Zapp & Green, 1989; Karn et al., 1991; Wingfield et al., 1991; Heaphy et al., 1990). Karn et al. (1991) and Wingfield et al. (1991) have demonstrated the ability of Rev to form filamentous structures in the absence and presence of RRE. Daly et al. (1989), Malim et al. (1990), and Heaphy et al. (1990) have determined that between 6 and 10 Rev molecules are capable of interaction with the RRE in the presence of excess nonspecific RNA *in vitro* although Cook et al. (1991) demonstrated specific binding of a single Rev protein to a subdomain of the RRE. It has been suggested by Malim and Cullen (1991) as well as Zapp et al. (1991) that Rev activity occurs through multimerization via the M4 domain of Rev, located in the N-terminal domain of the protein, proximal to the arginine-rich RNA binding domain. Zapp et al. (1991) have further concluded that multimerization is required for specific RRE binding. Daly et al. (submitted for publication), however, have shown that perturbations within the amino-terminal domain of Rev alter both the ability of the protein to multimerize as well as the ability to discriminate RRE from nonspecific RNA. Currently it is unclear whether these two activities are linked. Rev multimerization occurring only through the M4 domain might be expected to result in formation of a stable dimeric species. However, because Rev is capable of extensive oligomerization both in the absence and in the presence of RNA, it is likely that one or more additional domains within Rev may play a role in formation of protein–protein interactions. A series of Rev mutants generated by Malim et al. (1989) have been studied for effects on biological activity and are currently being investigated for perturbations of *in vitro* activity.

Mutational analysis of Rev has resulted in the generation of a class of Rev mutant (M10) possessing a transdominant phenotype (Malim et al., 1989). The transdominant phenotype of M10 has been attributed to an inability to interact with a host cellular factor, of which several have been revealed (Fankhauser et al., 1991; Ruhl et al., 1993). Extensive mutagenesis of the transactivation domain has subsequently revealed the presence of consensus amino acids required for biological activity (Venkatesh & Chinnadurai, 1990; Malim et al., 1991). This consensus sequence is thought to be required for direct binding to the host cellular factor. It is also possible that Rev activity at least in part depends upon the interaction of two or more Rev species interacting through the transactivation domain correctly placed on the RRE, thus permitting the formation of a composite cellular factor binding site. Transdominance might result from Rev monomers incorrectly interacting on the RRE, thereby unable to interact with the cellular protein. *In vivo* studies by Hope et al. (1992), which argue that transdominance occurs through formation of nonfunctional complexes between mutant and wild-type Rev, support the contention that this region of the monomeric protein does not interact directly with a host cellular factor. If this hypothesis is correct, then regions within the C-terminal or transactivation domain of Rev may function in generation of specific Rev–Rev interactions.

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Cochrane et al. (1989) and Hauber et al. (1988) demonstrated that Rev was capable of being phosphorylated through specific serine residues within the C-terminus, although mutagenesis studies showed that phosphorylation was not required for activity. Cochrane et al. (1989) created a Rev mutant lacking the C-terminal 25 amino acids and showed that it was biologically active *in vivo*. In addition, they were able to demonstrate binding of this mutant to the RRE *in vitro* (Olsen et al., 1990). It is possible that perturbation of the carboxy terminus of Rev may result in significant changes in the *in vitro* activity of the protein which are dispensable for biological activity and have therefore gone undetected. In order to more fully understand the functional relationships between the amino-terminal, transactivation, and carboxy-terminal domains, a series of Rev mutant proteins have been examined for RRE binding, multimerization properties, and biological activity *in vivo*. The results demonstrate that deletions within the C-terminus of Rev result in profound changes in multimerization properties but do not affect the *in vivo* activity of the protein. Furthermore, the function of Rev appears to be the result of the RRE-dependent binding of two interacting Rev proteins.

MATERIALS AND METHODS

Expression of Rev M10, Rev/Rex M10-16, Rev M9Δ14, and Rev M11Δ14. Rev mutants M10, M9Δ14, and M11Δ14 (Malim et al., 1989) were cloned for *Escherichia coli* expression by translational couple to the 5′-end of the *E. coli* β-glucuronidase gene carried by expression plasmid pREV2.1 as described previously for wild type Rev protein (Daly et al., submitted for publication). The Rev/Rex M10-16 hybrid carried on plasmid pIW9 (Weichselbraun et al., 1992) was also cloned into pREV2.1 for *E. coli* expression in a similar manner. Expression plasmids were transformed into *E. coli* strain RGN714 and grown in 10-L fermentation batches as previously described (Daly et al., submitted for publication).

Purification of Rev Mutant M9Δ14. The C-terminal deletion mutant Rev M9Δ14 was expressed in *E. coli* and purified by the following protocol. Cells were lysed by Dynomil treatment in buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM DTT, 5 mM EDTA, 1% PMSF, and 0.1 M NaCl. Following lysis, the mixture was centrifuged for 1 h at 25 °C at 8000 rpm. The supernatant from the centrifugation step was loaded onto a Q-Sepharose ion-exchange column equilibrated in 50 mM Tris-HCl, pH 8.5, 10 mM DTT, and 3 mM EDTA and the mutant protein eluted with a 0–1.5 M NaCl gradient in 50 mM Tris-HCl, pH 8.5, 10 mM DTT, and 3 mM EDTA. Fractions containing the mutant protein were concentrated by precipitation with 4 M NaCl. Protein was subsequently solubilized in buffer containing 8 M urea/50 mM Mes, pH 6.5, and loaded onto an S-Sepharose column equilibrated with 8 M urea/50 mM Mes, pH 6.5. The protein was eluted using a 0–2 M NaCl gradient. Fractions containing purified Rev M9Δ14 were pooled and quantitated by amino acid analysis, and purity was assessed by Coomassie staining following SDS–PAGE.

Purification of Rev M11Δ14. The C-terminal deletion mutant Rev M11Δ14, which lacks amino acids 92–112 of the native sequence, was expressed in *E. coli* and purified as follows. Cells were suspended in chilled lysis buffer containing 50 mM Mes, pH 6.5, 5 mM EDTA, 0.1% Triton X-100, 2 mM $MgCl_2$, 5 mM DTT, and 0.25 mM PMSF. The cell suspension was lysed by Dynomil treatment using a 0.6-L chamber at 4 °C. Following lysis, several 4000–8000-unit aliquots of DNase I (Sigma) were added to the suspension, and incubation proceeded with stirring at room temperature for 2 h. The suspension was subsequently centrifuged at 10 000 rpm at 4 °C for 2 h. The supernatant from the centrifugation step was decanted and the precipitate resuspended in lysis buffer for an additional extraction. Following resuspension, the solution was recentrifuged at 10 000 rpm at 4 °C for 1 h. The supernatants from the two centrifugation steps were then combined, and an equal volume of 100% ethanol was slowly added at 4 °C and precipitation proceeded overnight. The mixture was then centrifuged for 2 h at 10 000 rpm, and the resulting pellet was extracted in buffer containing 50 mM Mes, pH 6.5, 8 M urea, and 5 mM DTT. This extraction proceeded with stirring at room temperature until the pellets were fully resuspended. Following extraction, the solution was centrifuged for 12 h at room temperature at 10 000 rpm and the supernatant loaded onto a CM-Sepharose column equilibrated in CM column buffer (Tris-HCl, pH 7.6, 8 M urea, and 5 mM DTT). Protein was eluted with CM column buffer using a 0–1.2 M NaCl gradient. Fractions containing the mutant Rev M11Δ14 protein were pooled and further purified using C4 reverse-phase Vydac semipreparative HPLC. Protein was eluted using a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions containing the protein were lyophilized and resolubilized in 50 mM Mes (pH 6.5)/8 M urea for refolding. Protein purity as judged by SDS–PAGE Coomassie staining was greater than 90%.

Purification of Rev/Rex M10-16. Rev/Rex M10-16 was expressed in *E. coli* and purified as previously described (Weichselbraun et al., 1992). Cells were suspended in chilled lysis buffer containing 50 mM Mes, pH 6.5, 50 mM ε-aminocaproic acid, 5 mM EDTA, and 0.05% Triton X-100. The cell suspension was lysed by Dynomil treatment using a 0.6-L chamber at 4 °C. During cell lysis, 0.25 mM PMSF was added to the mixture. Following lysis, several 4000–8000-unit aliquots of DNase I (Sigma) and 2 mM $MgCl_2$ were added to the protein solution. Digestion proceeded for 2 h at 25 °C. Following digestion, the solution was centrifuged for 2 h at 10 000 rpm at 4 °C. The supernatant was brought to 50% ethanol with stirring on ice and incubated overnight during which time the protein precipitated. Following centrifugation of the precipitate at 10 000 rpm for 2 h at 4 °C, the pellet was extracted in buffer containing 50 mM Mes, pH 6.5, 8 M urea, 5 mM EDTA, and 5 mM DTT for 5 h at room temperature. The solubilized protein was centrifuged overnight at room temperature at 10 000 rpm and the supernatant loaded onto an S-Sepharose ion-exchange column equilibrated in 50 mM Mes, pH 6.5, 8 M urea, 5 mM EDTA, and 5 mM β-mercaptoethanol (S column buffer). Protein was eluted using a 0–1.3 M NaCl gradient in S column buffer, and fractions containing Rev/Rex M10-16 were pooled and concentrated by Amicon filtration (JM3 filter). The protein was subsequently loaded onto an Sepharose S-200 gel filtration column equilibrated in S column buffer. Fractions containing protein were pooled and refolded out of urea by S-Sepharose chromatography using a 0–1.6 M NaCl gradient in buffer containing 50 mM Tris-HCl (pH 7.6)/5 mM EDTA. Protein was analyzed by SDS–PAGE and determined by Coomassie staining to be greater than 95% pure.

Cleavage with CNBr/Purification. Rev/Rex M10-16 was dialyzed against several changes of distilled water, lyophilized, and resuspended in 70% formic acid. The protein was reacted with a 600-fold molar excess of CNBr for 24 h with inert gas passed over the reaction. The solution was lyophilized and washed twice by resuspension in distilled water. Following the second wash, the protein was resuspended in 0.1 M

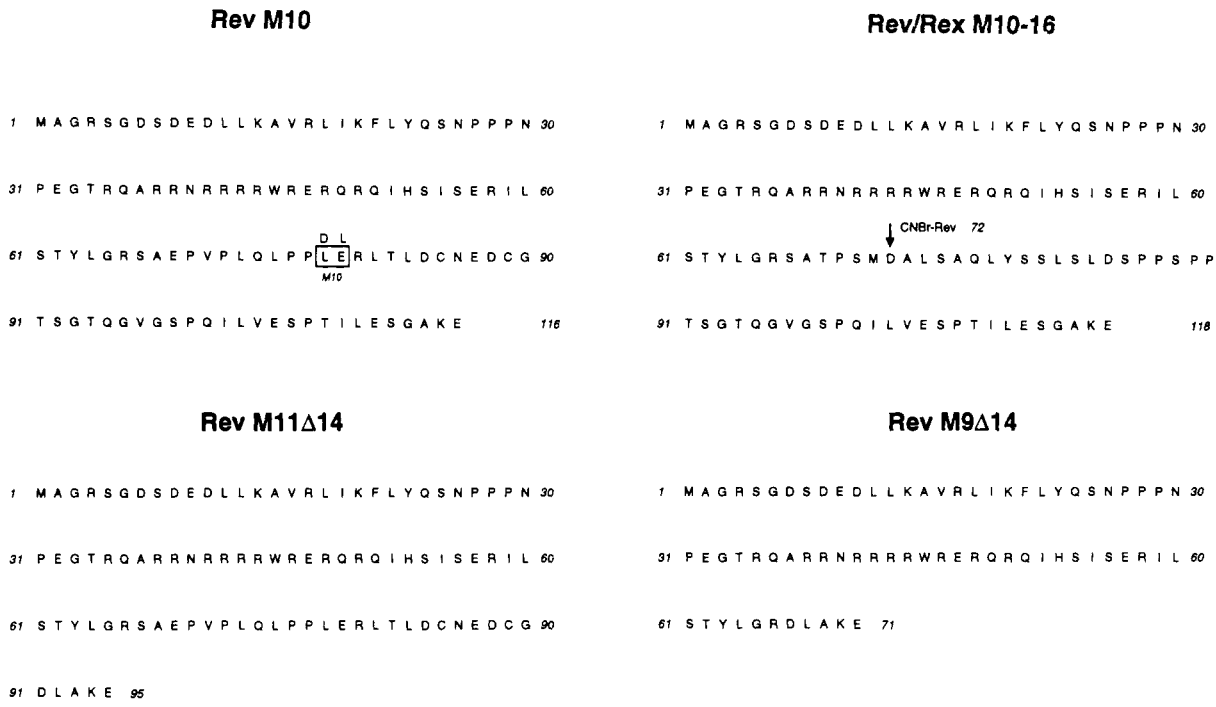

FIGURE 1: Amino acid sequences of the transactivation and C-terminal deletion mutant Rev proteins.

ethanolamine (pH 9.0)/8 M urea and loaded onto a Sepharose S-100 gel filtration column equilibrated with the same buffer. Fractions containing the truncated protein were either quantitated and added directly to RNA binding assays or subsequently dialyzed against 50 mM Mes (pH 6.5)/4 M urea. The dialysate from this step was diluted 1:4 with water after which the sample was subjected to dialysis. CNBr-cleaved Rev/Rex as well as Rev M9Δ14 and Rev M11Δ14 could be stably refolded in 0.2 mM Mes (pH 6.5)/16 mM urea.

Binding Studies. Gel shift and nitrocellulose filter binding experiments were performed as previously described unless otherwise noted (Daly et al., 1989). Dissociation rate experiments were performed using an initial ratio of protein to RRE of 8 to 1. Dissociation was initiated by addition of an excess of unlabeled RRE or poly(G) RNA.

Construction of Reporter and Expression Plasmids. The reporter plasmid p24RRE was constructed essentially as described by Lewis et al. (1990), with the gag/pol portion derived from the provirus pNL4-3 (Adachi et al., 1986). The expression plasmids SV40Rev, SV40M10, and SV40Rev/Rex M10-16 were made by PCR amplifying their coding sequences from the *E. coli* expression plasmids and cloning these into an SV40 expression vector. All sequences were verified by extensive sequencing of both DNA strands.

Transient Transfection of Cos 7 Cells with DNA. Cos 7 cells were kept in continuous culture in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS). One day prior to transfection, the confluent 15-cm plates of cells were split 1:2 into fresh plates. The next day the freshly confluent plates were trypsinized and the cells counted. A total of 1×10^7 cells were placed into 15-mL conical tubes, and the cells were pelleted. After aspiration of the media, DNA was added directly to the cell pellet. All samples contained 2 μg of p24RRE and either none of varying amounts of the expression plasmids; 225 μL of ice-cold RPMI 1640 (Gibco) containing 10% FBS was then added to the pellet. The cells were resuspended in this medium, transferred to a 4-cm electroporation cuvette (Bio-Rad), and incubated on ice for 10 min. Electroporation conditions were essentially those recommended by the manufacturer (960 μF, 0.25 KV, 400 Ω). The shocked cells were incubated on ice for 10 min followed by transfer to 10-cm dishes containing DMEM/10% FBS. Live cells were allowed to reattach for approximately 3 h. Subsequently, the medium was changed and cell debris removed. The cells were incubated until 65-h post-transfection at which time the medium was harvested.

Transient Transfection of Cos 7 Cells with Purified Protein. Cos 7 cells were transfected with p24RRE as described above, except that the shocked cells were plated in 6-well dishes containing 1 mL of DMEM/10% FBS. After the cells had adhered to the plastic, the medium was removed and replaced with 1 mL of DMEM without FBS but containing 100 μM chloroquine (Sigma). Protein was added to each well in equal volumes and identical buffer. Transfected cells were incubated for 5 h at which time the medium was removed, the cells were washed once with PBS, and 1 mL of DMEM/10% FBS was added. The cells were then incubated until 65-h post-electroporation, at which time the medium was harvested.

ELISA Analysis of Transfected Cells. Media sample volumes of 200, 20, and 2 μL were analyzed using a p24 ELISA kit (DuPont—NEN). Values were generated using the average of points which fell within the linear range of the assay, and compared to the curve generated using the p24 standards provided.

RNA Synthesis and Purification. RNA species used in the *in vitro* binding assays were synthesized using T7 RNA polymerase transcribed pBluescript constructs as previously described. [α-^{32}P]UTP-labeled RNA fragments were purified by electroelution following electrophoresis on a 5% TBE–polyacrylamide gel.

RESULTS

Purification of Transactivation and Carboxy-Terminal Rev Mutants. A series of Rev protein species containing mutations within either the transactivation or the carboxy-terminal domains were expressed in *E. coli* by translational coupling to the β-glucuronidase gene. The sites of mutations within the Rev sequence are depicted in Figure 1. Rev/Rex M10-16

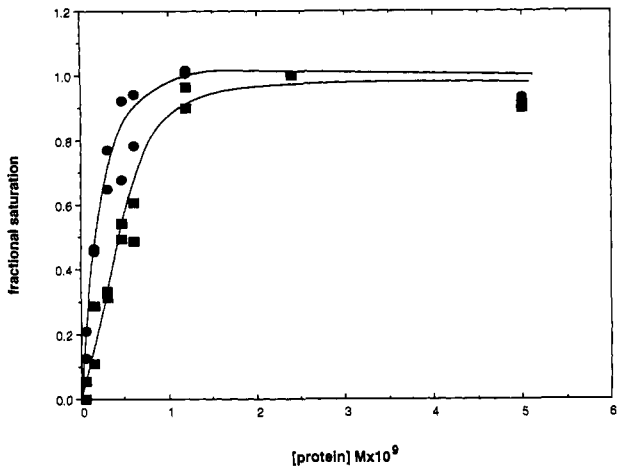

FIGURE 2: Direct nitrocellulose filter binding of M10 transactivation mutant protein to the RRE. Samples containing varying concentrations of M10 Rev mutant were incubated with ^{32}P-labeled RRE in the presence (■) or absence (●) of excess unlabeled MS2 competitor RNA (1.45×10^{-5} M base). Samples were incubated at room temperature for 30 min, followed by filtration through nitrocellulose membranes. Membranes were dried, and radioactivity bound to the filters was quantitated by scintillation counting.

was generated by replacement of the Rev transactivation domain for that of HTLV-I Rex protein by substitution of amino acids 69 through 91 of Rev with amino acids 75 through 99 of Rex (Weichselbraun et al., 1992). CNBr-Rev was generated by CNBr cleavage of Rev/Rex M10-16 at methionine-72, resulting in the generation of a truncated protein species of molecular weight of approximately 8000 containing four amino acids on the C-terminus derived from Rex. Rev M9Δ14 and Rev M11Δ14 result from internal deletions within the transactivation and C-terminus or C-terminus domains, respectively (Malim et al., 1989). In both cases, the C-terminal three amino acids from the native protein are included in the mutants. All of the protein species tested in the study were purified to greater than 90% purity through a series of ion-exchange and HPLC purification steps previously described.

Rev C-Terminal and Transactivation Mutants Bind to the RRE with High Affinity and Specificity. All of the mutant proteins were examined for the ability to bind to either sense or antisense RNA through direct nitrocellulose filter binding. The binding results of the M10 mutant are shown in Figure 2. In either the absence or the presence of unlabeled MS2 RNA, all of the protein species are able to bind to the RRE with an equilibrium binding constant of between 1×10^{-10} and 6×10^{-10} M (not shown). Only in the absence of unlabeled excess MS2 RNA was binding observed by the mutants to the antisense RNA species, indicating that these proteins recognized antisense RNA with high affinity. Therefore, sense and antisense RNA binding of Rev M10, Rev/Rex M10-16, CNBr-Rev, Rev M9Δ14, and Rev M11Δ14 all appear equivalent to that of wild-type Rev. Mutations within these domains do not appear to affect RNA binding characteristics.

The ability of these C-terminal and activation domain mutants to discriminate the RRE relative to antisense RNA using MS2 RNA as competitor was examined through the competition nitrocellulose filter binding assay (Figure 3). All of the mutants examined exhibited at least a 2000-fold preference for the RRE relative to the antisense RNA, demonstrating that unlike the amino-terminal-perturbed Rev species, these Rev proteins possess all of the determinants necessary to discriminate the RRE from nonspecific RNA.

C-Terminal Rev Mutants Display Reduced Multimerization on the RRE in Vitro. Each of the mutants was

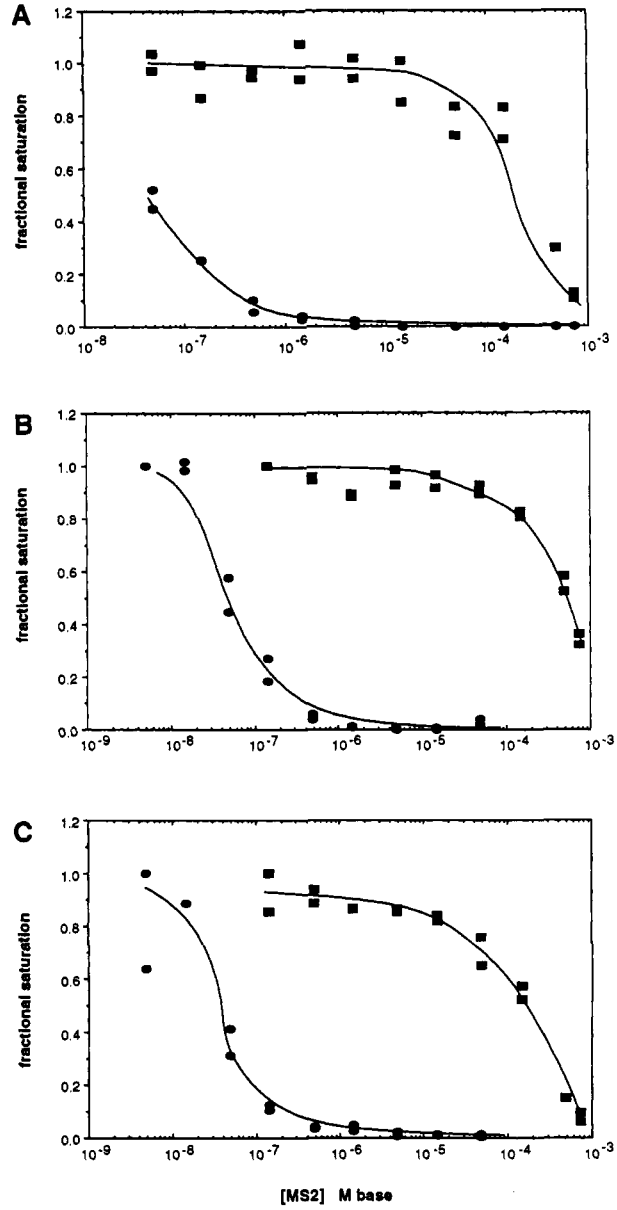

FIGURE 3: Preference of transactivation and C-terminal deletion Rev mutants for RRE binding relative to antisense RNA using MS2 as the competitor RNA species. Protein at constant concentration was added to ^{32}P-labeled RRE or antisense RNA in the presence of varying concentrations of unlabeled competitor RNA. Results were plotted as the fraction of bound protein to labeled RNA at each competitor concentration relative to bound protein in the absence of competitor. (A) M10 Rev: RRE (■); antisense RNA (●). (B) Rev M11Δ14: RRE (■); antisense RNA (●). (C) CNBr-Rev: RRE (■); antisense RNA (●).

subsequently examined in the gel shift assay for the ability to interact with the RRE. Figure 4A shows that the band pattern generated for the M10 mutant protein is equivalent to that of the wild-type protein, with at least seven discrete protein-dependent bands being apparent. As demonstrated in Figure 4B, this binding is highly specific for the sense RRE relative to the antisense RNA. Similar results were observed for the Rev/Rex M10-16 protein, suggesting that perturbations within the transactivation domain do not affect the multimerization pattern on the RRE (Figure 4C). When the C-terminal deletion mutants were examined in the assay and compared to wild-type Rev, the ability to multimerize on the RRE was significantly reduced (Figure 5A,B). Despite the decreased size of the Rev M11Δ14 and CNBr-Rev proteins, the results demonstrate that the complexity of the banding pattern relative

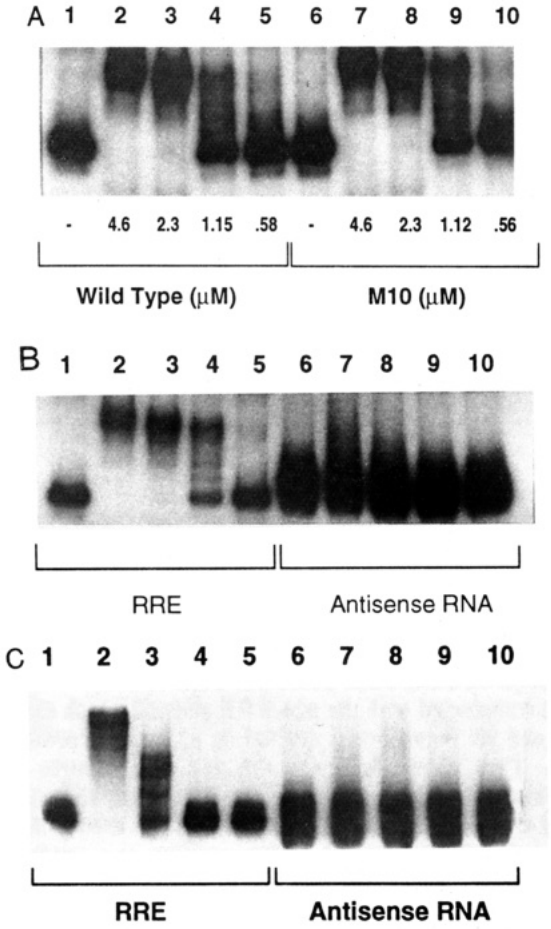

FIGURE 4: Gel shift analysis of M10 Rev and Rev/Rex M10-16 binding to RRE. (A) Comparison of wild-type Rev to M10 Rev at comparable concentration. (B) Binding of M10 Rev to RRE and antisense RNA under identical conditions. In all samples, unlabeled excess tRNA was added to abolish the nonspecific component of the binding interaction. M10 Rev concentrations for panel A were as follows: lanes 1 through 6, none; lane 7, 4.6 μM; lane 8, 2.3 μM; lane 9, 1.12 μM; lane 10, 0.56 μM. Wild-type Rev concentrations for panel A were as follows: lanes 1 and 6 through 10, none; lane 2, 4.6 μM; lane 3, 2.3 μM; lane 4, 1.15 μM; lane 5, 0.58 μM. M10 Rev concentrations for panel B were as follows: lanes 1 and 6, none; lanes 2 and 7, 4.6 μM; lanes 3 and 8, 2.3 μM; lanes 4 and 9, 1.12 μM; lanes 5 and 10, 0.56 μM. (C) Gel shift analysis of Rev/Rex M10-16 binding to either RRE or antisense RNA. Protein concentrations were as follows: lanes 1 and 6, none; lanes 2 and 7, 0.75 μM; lanes 3 and 8, 0.38 μM; lanes 4 and 9, 0.19 μM; lanes 5 and 10, 0.1 μM.

to the wild-type protein has been reduced. These results, in conjunction with the filter binding data, argue that decreased multimerization of Rev has no affect on the ability of the protein to recognize the primary binding site on the RRE.

The lower molecular weight of the Rev M9Δ14, CNBr-Rev, and Rev M11Δ14 proteins resulted in smaller band shifts of the RRE–protein complexes in the gel shift assay which were difficult to analyze. To visualize the ability of these Rev C-terminal truncation mutants to multimerize, gel shift assays using stem II RNA were performed. Stem II is a 66-nucleotide RNA fragment containing the determinants required for specific Rev binding (Cook et al., 1991). A comparison of the binding of Rev M11Δ14 (Figure 6A, lanes 2–5) with the binding of wild-type Rev (Figure 6B, lanes 7–10) to stem II RNA shows the protein-dependent band patterns to be nearly identical. The R_f values for the bands generated in the presence of Rev M11Δ14 (0.71, 0.75, and 0.81) relative to unbound stem II RNA compare favorably to those observed for wild-type Rev binding to the RNA (R_f = 0.60, 0.64, and 0.71) and indicate a similar basic pattern. Differences between the

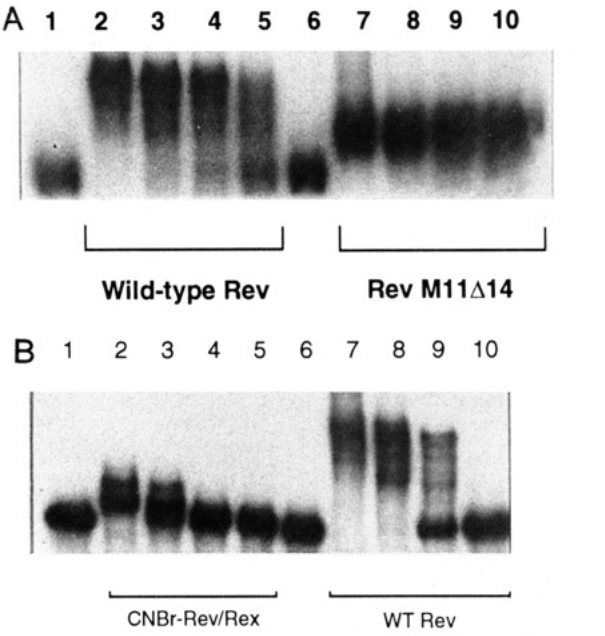

FIGURE 5: Gel shift analysis of the interaction of C-terminal deletion mutants with RRE. (A) Comparison of wild-type Rev and Rev M11Δ14. (B) Comparison of wild-type Rev and CNBr-cleaved Rev/Rex. Protein concentrations were varied as follows: lanes 1 and 6, none; lanes 2 and 7, 4 μM; lanes 3 and 8, 2 μM; lanes 4 and 9, 1 μM; lanes 5 and 10, 0.5 μM.

patterns produced by Rev M11Δ14 and wild-type Rev may be attributed to the decreased size and charge (loss of glutamic acid at positions 105 and 112) of the truncation mutant.

In contrast, the comparison between the binding of Rev M11Δ14 and Rev M9Δ14 in the presence of stem II RNA demonstrated a qualitative difference in the band pattern (Figure 6A). For the M9Δ14 mutant, two separate protein-dependent bands were observed at R_f values of 0.77 and 0.87, the predominance of which was dependent on either protein or stem II concentration (not shown). The pattern observed for the Rev M9Δ14 mutant which appears as two discrete bands in the gel shift assay was significantly different from the triplet pattern observed for the Rev M11Δ14 mutant. The CNBr-Rev, which is slightly smaller than Rev M9Δ14, produced shifted bands at R_f values of 0.78 and 0.89 relative to unbound RNA (Figure 6C) which were identical to the patterns produced by the Rev M9Δ14 protein. The results suggest either that a different number of 11Δ14 and 9Δ14 proteins interact with the stem II RNA or that perhaps differences in Rev–Rev interactions leading to differential folding of the RNA are being monitored by this technique. The results, however, suggest that two independently acting proteins such as Rev M9Δ14 which lack the transactivation domain are capable of specific binding to stem II RNA.

Perturbation of the Rev Transactivation Domain Affects Dissociation from the RRE. Through the use of dissociation rate experiments under pseudo-first-order conditions, it has been observed that at protein:RRE ratios greater than 1:1, the $T_{1/2}$ for wild-type Rev was approximately 25 min, suggesting that the Rev–RRE interaction is highly stable (Daly et al., 1993). Following binding of a single Rev protein to the RRE, the protein was observed to dissociate from the RRE with a $T_{1/2}$ that was approximately 9 min, implying that binding of multiple protein monomers results in stabilization of the protein–RNA interaction. The dissociation rates for the C-terminal deletion mutants were examined for perturbations in the off-rate. At protein monomer:RRE molar ratios of 8:1, the Rev 11Δ14 mutant was observed to dissociate from

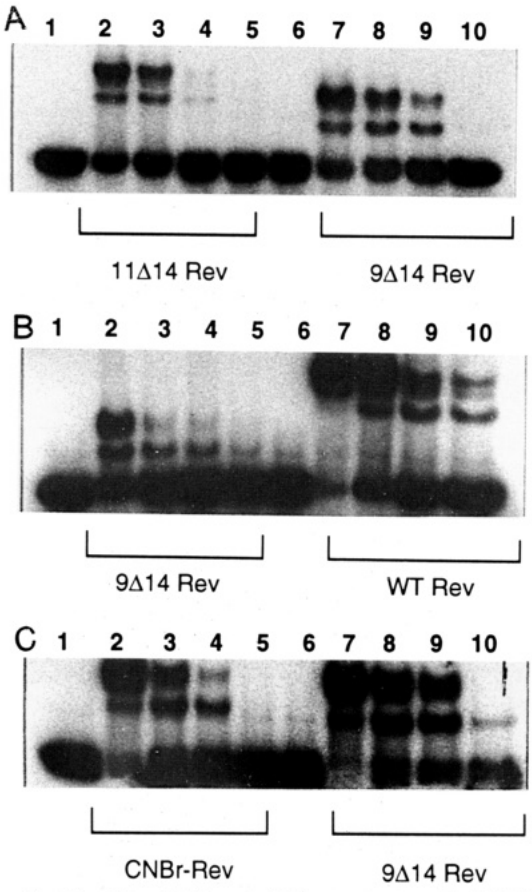

FIGURE 6: Titration of C-terminal Rev mutants with ^{32}P-labeled stem II RNA. (A) Comparison of binding of Rev M11Δ14 with Rev M9Δ14 to stem II RNA in the presence of excess tRNA. Protein concentrations were as follows: lanes 1 and 6, none; lanes 2 and 7, 1.6 μM; lanes 3 and 8, 0.8 μM; lanes 4 and 9, 0.39 μM; lanes 5 and 10, 0.2 μM. (B) Comparison of Rev M9Δ14 and wild-type Rev binding to stem II RNA. Protein concentrations were as follows: lanes 1 and 6, none; lanes 2 and 7, 10 μM; lanes 3 and 8, 5 μM; lanes 4 and 9, 2.5 μM; lanes 5 and 10, 1.25 μM. (C) Comparison of CNBr-cleaved Rev/Rex and Rev M9Δ14 binding to stem II RNA. Protein concentrations were as follows: lanes 1 and 6, none; lanes 2 and 7, 12 μM; lanes 3 and 8, 6 μM; lanes 4 and 9, 3 μM; lanes 5 and 10, 1.5 μM.

the RRE with a $T_{1/2}$ of approximately 20–25 min, a result equivalent to that which was observed for wild-type Rev. The result suggests that binding of the first two Rev monomers is sufficient to fully stabilize the complex on the RRE. The subsequent binding of an additional five or six Rev monomers does not contribute to an increase in the $T_{1/2}$. As shown in Figure 7, the off-rate from the RRE for the CNBr-Rev species at molar ratios of 8:1 was approximately 8 min, equivalent to dissociation of a single Rev protein from the RRE. The results demonstrate that removal of determinants within the putative transactivation domain (M9–M11) alters RRE binding characteristics, suggesting an additional function for this domain of the protein. Rev M9Δ14 displayed a similar dissociation rate from the RRE as the CNBr-Rev protein (not shown). Although two Rev M9Δ14 or CNBr-Rev protein monomers are capable of binding to the RRE, the 25-min $T_{1/2}$ dissociation rate observed for both wild-type and Rev M11Δ14 was not apparent. Alternatively, the results are consistent with dissociation of a single Rev monomer, or perhaps two noninteracting monomers from the RRE, suggesting that this region of the protein functions at least in part as an RRE-dependent Rev–Rev interaction domain.

Biological Activity of Transactivation and C-Terminally Truncated Rev Mutants. The biological activity of the

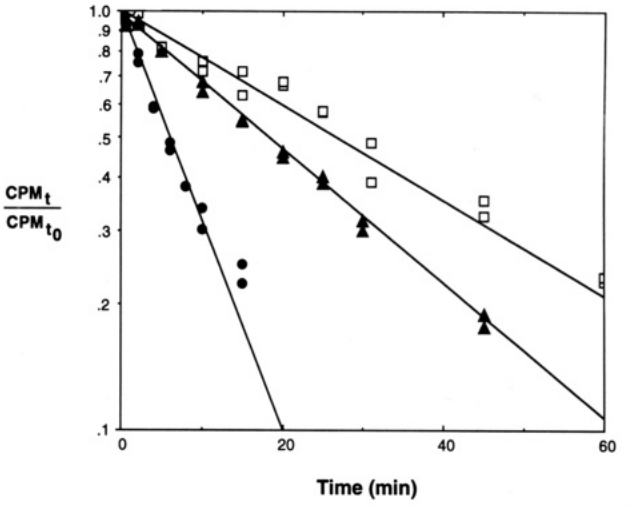

FIGURE 7: Dissociation rate of transactivation and C-terminal deletion mutant proteins from RRE at an initial binding molar ratio of 8 protein molecules per 1 RRE fragment. (□) Wild-type Rev; (▲) Rev M11Δ14; (●) CNBr-cleaved Rev/Rex.

C-terminal deletion and transactivation domain mutants was investigated using a cell-based p24 ELISA assay. Cos 7 cells were transfected with the p24-RRE plasmid which expresses gag and pol polypeptides (including p24) in the presence of Rev. Thus, cotransfection of the cells with a source of Rev in *trans* will result in the production of measurable amounts of p24. An ELISA kit (NEN/DuPont) was used to measure p24 secreted into the cell culture medium as a result of the presence of Rev or Rev mutants in *trans*.

Dose response curves were generated for p24 expression in the presence of 0.2–5.0 ng of cotransfected plasmids pSV40-Rev, pSV40-M10, and pSV40-Rev/Rex M10-16 (Figure 8A). pSV40-Rev transfection resulted in a linear dose response of p24 across the range of plasmid added. pSV40-M10 transfection did not activate p24-RRE in *trans*, and pSV40-Rev/Rex M10-16 activity was approximately 10-fold reduced relative to wild-type Rev, in agreement with previous results (Malim et al., 1989; Weichselbraun et al., 1992). Western blots of lysed cells probed with polyclonal anti-Rev antisera were used to confirm that all three plasmids were expressing protein at equivalent levels (data not shown).

The cell-based assay was also used as a means of monitoring transdominance through a series of experiments in which Rev wild-type and mutant plasmids were incubated with p24-RRE-transfected cells. The transdominant effect of pSV40-M10 was measured at a 1:1 ratio of pSV40-M10 to pSV40-Rev along the previously established dose response curve for pSV40-Rev. The effect of transdominance was to decrease Rev activity in the range of 40–60% (Figure 8B). Similarly, pSV40-M10 was able to decrease the activity of pSV40-Rev/Rex M10-16, further confirming that this mutant possesses a strongly transdominant phenotype. These observations are consistent with previously published reports (Malim et al., 1989). M10 has previously been shown to be transdominant over HTLV-III Rev and HTLV-I Rex protein (Weischelbraun et al., 1992), suggesting that this domain in Rex is capable of at least partially substituting for that of Rev *in vivo*.

Dose response curves were generated for the transactivation of p24 in the presence of 25–200 μg of purified Rev, M10, and Rev/Rex M10-16 proteins (Figure 9A). Results were similar to those obtained using the SV40 expression plasmids. Western blot analysis was used to confirm that similar amounts of purified protein were transfected into the cells (data not shown). Transfection of purified wild-type Rev, M10 Rev,

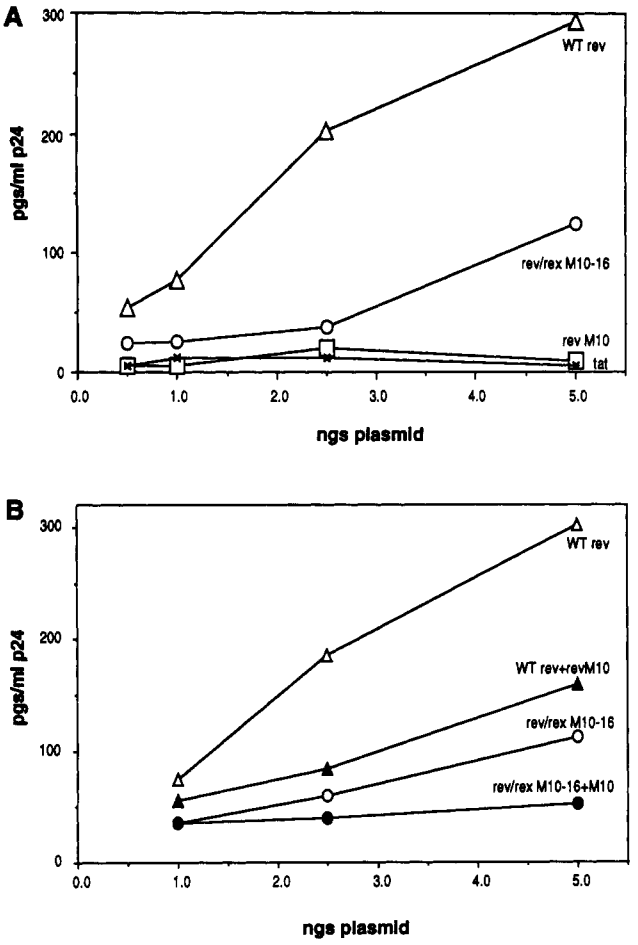

FIGURE 8: Cell-based assay studies. (A) Dose response curves of plasmid transfection. Cos 7 cells were electroporated with excess p24RRE and increasing amounts of the SV40 expression plasmids. Three-days posttransfection, the presence of p24 in the media was measured by ELISA assay. Expression plasmids: wild-type Rev (△); Rev/Rex M10-16 (○); Rev M10 (□); Tat (×). (B) Transdominance experiments using cotransfected plasmids. Cos 7 cells were electroporated with excess p24RRE and with SV40Rev and SV40M10 in equal amounts across the demonstrated linear range of SV40-driven expression of Rev. Three-days posttransfection, the presence of p24 in the media was measured by ELISA assay. Expression plasmids: (△) wild-type Rev; (▲) wild-type Rev + Rev M10; (○) Rev/Rex M10-16; (●) Rev M10 + Rev/Rex M10-16.

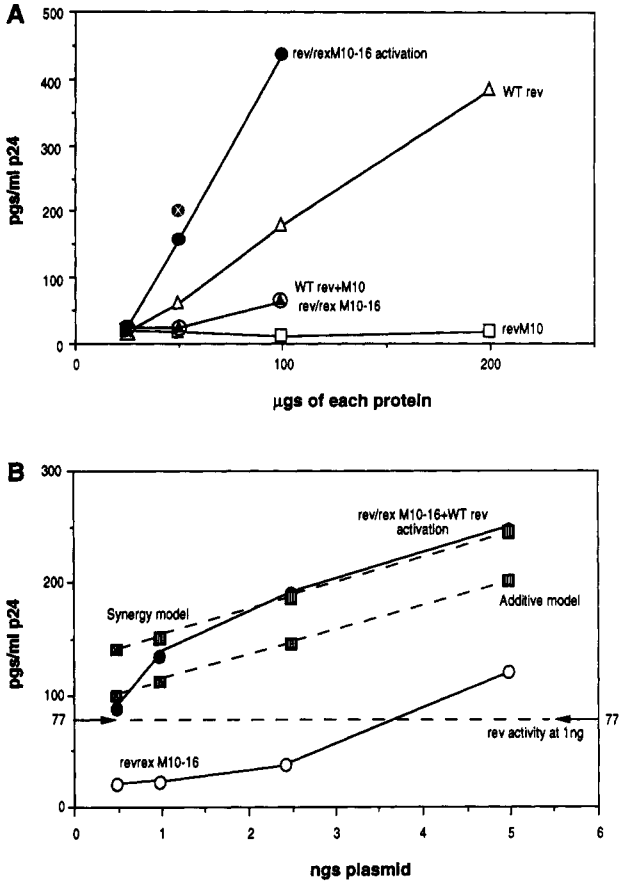

FIGURE 9: (A) Transactivation and transdominant effects of adding purified proteins to p24RRE-transfected cells. Cos 7 cells were electroporated with excess p24RRE. After 24 h, the cells were incubated for 5 h with purified proteins in the presence of chloroquine. Forty-eight-hours postincubation, the presence of p24 in the media was measured. Dose response curves: (△) 25–200 μg of wild-type Rev; (●) 25–100 μg of Rev/Rex M10-16 + 25–100 μg of wild-type Rev; (○) 25–100 μg of Rev/Rex M10-16 alone; (▲) 25–100 μg of M10 Rev + 25–100 μg of wild-type Rev; (□) 25–200 μg of M10 Rev alone; (x inscribed in solid circle) 50 μg of wild-type Rev + 100 μg of Rev/Rex M10-16. (B) Transactivation experiments using cotransfected plasmids. Cos 7 cells were electroporated with excess p24RRE and the expression plasmids SV40Rev/Rex M10-16 and SV40Rev. pSV40Rev/Rex M10-16 was kept constant at 1 ng; pSV40Rev amounts varied from 0.5 to 5 ng. Three-days posttransfection, the presence of p24 in the media was measured. The data are compared to predicted curves representing either additive or synergistic effects on p24 generation. Dose response curves: (●) 0.5–5 ng of pSV40Rev/Rex M10-16 in the presence of 1 ng of pSV40Rev; (○) 0.5–5 ng of pSV40Rev/Rex M10-16; (lined open squares) predicted curves for additive or synergistic effects of cotransfection. The line at 77 pg/mL p24 denotes wild-type Rev activity at 1 ng of pSV40Rev.

or Rev/Rex M10-16 was performed to test the ability of these proteins to elicit p24 production from Cos 7 cells. As shown in Figure 9A, purified wild-type Rev was active in p24 production in a dose-dependent manner. M10 Rev was inactive under identical conditions. Rev/Rex M10-16 displayed approximately one-tenth the activity as that of wild-type Rev, equivalent to the results obtained using plasmid constructs. At equimolar ratios, Rev M10 was observed to significantly decrease the activity of the wild-type Rev, demonstrating that the purified mutant is capable of exerting a transdominant phenotype.

Synergistic Activation of Rev/Rex with Wild-Type Rev. A synergistic increase of the purified mutant protein Rev/Rex M10-16 activity was measured using the cell-based assay upon cotransfection with wild-type Rev protein. Rev/Rex M10-16 and wild-type Rev added together to cells cotransfected with p24RRE elicited a response greater than the sum of the proteins added individually (Figure 9A). At all doses tested, and in multiple experiments, the effect of mixing Rev/Rex M10-16 and Rev wild-type at a 1:1 ratio resulted in a level of activity generated which was not additive (Rev wild-type activity + Rev/Rex M10-16 activity) but rather was synergistic (twice Rev wild-type activity). The addition of further amounts of Rev/Rex M10-16 above the amount of Rev wild-type resulted in a further, but additive, increase in activity. This result suggested that Rev wild type was able to rescue the activity of the mutant protein to that of wild-type protein levels (Figure 9A). Western blot analysis of p24-RRE-transfected Cos 7 cells 48 h after the addition of the purified proteins showed that equivalent amounts of protein were present (data not shown). In order to demonstrate that this increase in Rev/Rex M10-16 activity was not due to Rev wild type facilitating transport into the cell and/or the nucleus, this coactivation phenomenon was confirmed using the expression plasmids pSV40-Rev and pSV40-Rev/Rex M10-16 (Figure 9B). Plasmids were cotransfected with p24-RRE,

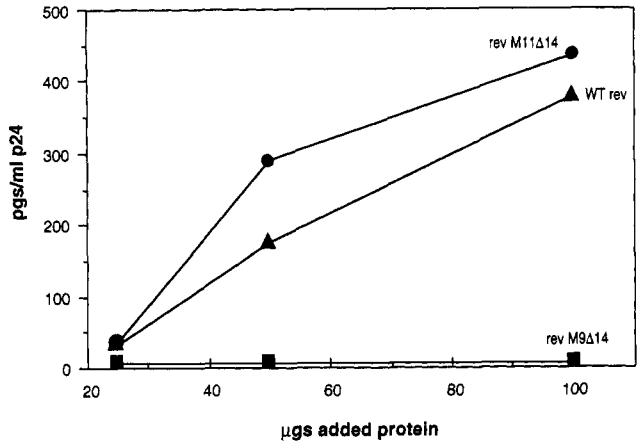

FIGURE 10: Titration of purified C-terminal deletion mutant proteins Rev M9Δ14 and Rev M11Δ14. Cos 7 cells were electroporated with excess p24RRE. After 24 h, the cells were incubated for 5 h with purified protein in the presence of chloroquine. Forty-eight-hours postincubation, the presence of p24 in the media was measured by ELISA. Rev M9Δ14 (■); Rev 11Δ14 (●); wild-type Rev (▲).

dose response curves were generated, and mixing experiments were performed, with results similar to those seen with the purified proteins. In these experiments, the amount of pSV40-Rev was held constant at 1 ng while pSV40-Rev/Rex M10-16 was varied across the linear range for wild-type activity (0.2–5 ng). p24 levels were measured at each point, and were compared to the level measured using pSV40-Rev/Rex M10-16 alone at each amount, pSV40-Rev at 1 ng addition of plasmid, a theoretical curve assuming a mechanism of interaction involving additive activity, and a theoretical curve assuming a mechanism involving synergistic activity. The results in Figure 9B suggest that the data best fit the theoretical model of synergistic coactivation rather than a model involving additive activities.

Rev M11Δ14 Is Biologically Active. Rev mutant proteins M9Δ14 and M11Δ14 were also tested in the cell-based assay for biological activity. Purified proteins were transfected into Cos 7 cells under previously described conditions. The Rev M9Δ14 mutant displayed no biological activity at any of the concentrations studied (Figure 10). This result is consistent with a protein species capable of high-specificity RRE binding which possesses a transdominant phenotype, similar to that of the M10 Rev mutant protein. The M11Δ14 mutant, however, demonstrated p24 activity equivalent to that of wild-type Rev in a dose-dependent manner, suggesting that although the ability of this protein to polymerize on the RRE is abolished, the biological activity of this species remains intact. The biological activity of Rev, therefore, is not predicated on the ability of the protein to saturate the RRE. Rather, two or three protein monomers appear necessary for biological activity.

DISCUSSION

A series of transactivation and carboxy-terminal Rev mutants were expressed, purified, and biochemically characterized for RRE binding activity, multimerization, and biological activity. Although several of these proteins were previously examined *in vivo* for activity, limited *in vitro* biochemical characterization had been completed (Malim et al., 1989; Weichselbraun et al., 1992). All of the protein species tested were expressed at high levels, facilitating purification to greater than 90% purity. The transactivation mutants M10 and Rev/Rex M10-16 displayed solubilities and RNA binding characteristics analogous to wild-type Rev. The C-terminal mutants, however, showed low solubility in high salt and maximal solubility in solution of low ionic strength. Unlike the amino-terminal mutants, all of these carboxy-terminal mutants displayed very high binding specificity (ability to discriminate RRE from nonspecific RNA) and affinity.

We have previously observed in the gel shift assay that at least seven discrete Rev-dependent bands were generated in the presence of labeled RRE. This pattern was defined as a multimerization of the protein on the RRE and may be distinct from the RRE-independent polymerization phenomenon observed by Karn et al. (1991) and Wingfield et al. (1991). In this study using gel shift assays, it has been demonstrated that removal of the carboxy-terminal domain (M11–M14) of Rev results in a protein which is unable to fully multimerize on the RRE. In the gel shift assay, Rev M11Δ14 was able to bind to the stem II RNA and produced a banding pattern similar to that of wild-type Rev. At present, it is unclear whether the third band observed following the interaction of Rev M11Δ14 with stem II RNA represents a unique species containing three protein monomers bound to a single stem II RNA fragment or is a result of conformational changes within the stem II RNA fragment following binding of two Rev M11Δ14 monomers. However, it is apparent that the banding pattern for Rev M11Δ14 differs significantly from that of Rev M9Δ14 which displayed only two discrete bands in the presence of stem II RNA. The observed difference between Rev 11Δ14 and 9Δ14 binding patterns demonstrates that removal of the transactivation domain results in a protein species which possesses altered multimerization activity which could be detected upon binding to the stem II RNA in the gel shift assay. Because of the highly specific nature of the RRE binding of the Rev M9Δ14 mutant, it is probable that the observed changes in the gel shift pattern are not a result of a misfolded Rev M9Δ14 protein species. This result suggests that the transactivation domain is not solely involved in direct contact with the host cellular factor but may also function in RRE-dependent Rev–Rev protein interactions. The reduction of wild-type Rev-like multimerization on the RRE observed with Rev M11Δ14 strongly suggests that binding of more than two or possibly three Rev proteins will not significantly affect Rev biological activity and that the requirement of complete multimerization (seven or eight Rev monomers) on the RRE for specific recognition and biological activity is not supported. Although we cannot address whether the truncated proteins are unable to polymerize and form filaments *in vitro*, it is clear that the extent of Rev multimerization on the RRE has been reduced.

The cell-based biological assay has been used to quantitate the effect of the transdominant mutant M10 on the activity of wild-type Rev. The results from these transdominant experiments were fully consistent with results from Malim et al. (1991). The results obtained using either transfected plasmid or purified protein were equivalent. The results obtained from the transfection of Rev/Rex M10-16 in the absence or presence of wild-type Rev demonstrated that this protein, though able to bind to the RRE with high affinity and specificity, is impaired in its ability to elicit full biological activity. Surprisingly, the presence of wild-type Rev appeared to rescue the activity of the Rev/Rex M10-16 mutant, suggesting that at least two Rev monomers must interact to promote biological activity. Furthermore, since Rev/Rex M10-16 possesses the consensus leucine motif proposed by Malim et al. (1991), we would propose that the rescued activity

occurs via interaction with this sequence. The M10 Rev mutant possesses a transposition of one of the leucine residues within this domain and might therefore be unable to form the requisite Rev–Rev interaction. These results, which argue that two interacting proteins bound to the RRE are required for activity, are consistent with results from Malim and Cullen (1991) as well as Zapp et al. (1991), who have previously proposed a functional Rev dimer being required for biological activity.

The transfection assay results implicate the M9–M11 domain of Rev as a putative dimerization domain and are consistent with the *in vitro* binding data which demonstrate that perturbation of the M9–M11 domain results in differences in gel shift band patterns as well as dissociation rates from the RRE. Since the Rev M11Δ14 mutant which is only lacking the carboxy terminus possesses a dissociation rate equivalent to that of the wild-type Rev, it may be concluded that the Rev M9Δ14 mutant requires the activation domain for wild-type RRE dissociation. Furthermore, as previously shown, the dissociation rate for a single wild-type Rev monomer from the RRE was equivalent to that of eight Rev M9Δ14 mutant proteins, suggesting that Rev–Rev interactions enhance stabilization of the proteins bound to the RRE.

The carboxy-terminal domain of Rev (M11–M14), which appears to be dispensable for activity, may be responsible for much of the complexity observed in this system *in vitro* and is likely involved in RRE-independent Rev–Rev interactions. In the presence of this domain (M11–M14), an additional type of Rev–Rev interaction occurs, thereby making detection of RRE-dependent specific Rev–Rev interactions difficult. M10 Rev, for example, appears to interact with the RRE in the gel shift assay in a manner which is analogous to wild-type Rev and dissociates from the RRE with an off-rate comparable to that of wild type. We speculate whether a C-terminally truncated form of Rev M10 would display wild-type or Rev M9Δ14-like dissociation rates from the RRE. In any event, separation of domains of activity on Rev would be useful in understanding how this protein functions.

On the basis of the results presented here, as well as previous studies, a mechanism of action for this protein is emerging. The arginine-rich nucleolar localization domain provides nearly all of the free energy of binding of Rev to the RRE through a number of contacts (1×10^{-9} M). Arginines, however, have previously been shown to interact with high affinity with guanine residues, an observation which explains why Rev interacts with high affinity with many RNA fragments (Helene, 1977; Porschke, 1978; Seeman et al., 1976). Arginines have also been shown to interact with phosphate groups on bulged or looped regions of RNA (Calnan et al., 1991). The fundamental discrepancy of how a protein with equivalent affinities can discriminate the RRE from large excesses of nonspecific binding sites *in vivo* may be explained through this interaction. The high affinity of the arginines for guanine residues may play a role in searching for the correct target binding sequence on the nascent mRNA. A calculation of the off-rate of a Rev arginine-containing peptide based on the measured equilibrium dissociation constant and diffusion-controlled binding suggests that the off-rate from nonspecific RNA would be very fast compared to that of the intact protein from the RRE (Daly et al., 1993). Alternatively, conformational changes within the RRE may accompany binding of the native protein, thereby stabilizing the interaction. In the absence of the amino terminus, which appears to stabilize the correct structure of the RNA binding domain, the peptide will dissociate and reassociate rapidly, perhaps reflecting changes in the structure of the peptide. Given the correct context (an intact amino terminus), the arginine-containing peptide is stabilized in a conformation suitable for binding to the RRE with the off-rate reflecting the observed specificity (discrimination) in binding.

The M4 domain of Rev also is a multimerization domain (Malim & Cullen, 1991). However, because two different activities are affected by mutations within this region (dimerization and RRE discrimination), characterization has been difficult (Daly et al., 1993). M4 Rev is a mildly transdominant mutant, reflecting a decreased discrimination of the RRE, as well as inhibited multimerization. Phenotypically, a lack of discrimination would not be transdominant. However, coupled with the failure to adequately dimerize (analogous to Rev M9Δ14), this mutant would display a weakly transdominant phenotype. Therefore, M4 Rev and Rev M9Δ14 may represent two forms of the same type of mutation. That is, both affect dimerization of Rev in an RRE-dependent manner, the difference between the mutants being that Rev M4 is less able to discriminate the RRE from nonspecific RNA. One model which supports this hypothesis would anticipate the interaction of the M4 region of one Rev with the M9-11 region of the second. This would permit two regions of interaction on each protein monomer, and the symmetry would allow the formation of a composite cellular factor binding site with an extended leucine motif involved in the interaction. This model is supported by all the *in vivo* mutational analysis data as well as recent cellular factor cross-linking studies which suggest that dimerized Rev (through modified cysteine residues) will interact with the cellular factor (Malim et al., 1989; Ruhl et al., 1993).

Finally, the carboxy-terminal domain (M11–M14) of Rev appears to play no role in the biological activity of the protein. However, it appears to affect multimerization of Rev on the RRE. This form of Rev–Rev interaction may affect the stability of complexes or have other implications for protein function. Further studies may permit understanding of the activity of this domain.

The ability of wild-type Rev to multimerize on the RRE may somehow modulate activity or perhaps stabilize the highly folded RRE containing messenger RNA. Previously several groups have observed that the carboxy-terminal domain of Rev is a putative target for phosphorylation. This phenomenon was deduced to be dispensable for activity. Cochrane et al. (1989) also reported that loss of the C-terminal 25 amino acids of Rev resulted in no change in the biological activity of the protein. Zapp et al. (1991) also suggest that the C-terminus of Rev is unimportant for biological activity. In this study, it has been shown that removal of the carboxy-terminal domain of Rev alters multimerization on the RRE but has no effect on the biological activity of the protein. Since reduced multimerization appears to play no role in biological activity, perhaps perturbations within the C-terminus affect later stages of Rev function including stabilization of RRE conformation or dissociation from the RRE, thereby permitting translation of the viral message.

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