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Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: A dual function for an arginine-rich binding motif

(mRNA export/pre-mRNA splicing/RNA binding protein/virus expression)

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ABSTRACT **The Rev protein of human immunodeficiency virus type 1 is a sequence-specific RNA binding protein that is essential for viral replication. Here we present evidence that Rev is a stable oligomer both *in vitro* and *in vivo*. Analysis of Rev mutants indicates that oligomerization is essential for RNA binding and hence Rev function. The oligomerization and RNA binding domains overlap over 47 amino acids. Within this region is a short arginine-rich motif found in a large class of RNA binding proteins. Substitution of multiple residues within the arginine-rich motif abolishes oligomerization, whereas several single-amino-acid substitution mutants oligomerize but do not bind RNA. Thus, Rev's arginine-rich motif participates in two distinct functions: oligomerization and RNA binding.**

The human immunodeficiency virus type 1 Rev protein acts posttranscriptionally to increase selectively the cytoplasmic levels of the gag–pol and env mRNAs (1–5). A specific RNA region that is required for Rev to function (*R*ev *R*esponse *E*lement; RRE) has been mapped within the gag–pol and env mRNAs (3, 6, 7). Rev is a sequence-specific RNA binding protein that interacts directly with the RRE (8–14). This sequence-specific RNA binding activity explains the basis for Rev's selective action. Rev is a member of a class of RNA binding proteins characterized by the presence of an arginine-rich motif (15, 16).

In this report we study the RNA binding of Rev in further detail and analyze Rev mutants previously characterized *in vivo*. We find that Rev binds to RNA as an oligomer and is a stable oligomer in the absence of the RRE. We then map the domains required for oligomerization and RNA binding. Our results indicate that oligomerization is essential for Rev function.

MATERIALS AND METHODS

Restriction enzymes were from Promega and GIBCO/BRL. RNases T1 and A, DNase I, and heparin-Sepharose were purchased from Pharmacia. DEAE-Sepharose CL-6B was from Sigma. Glutaraldehyde was from Aldrich. Dimethyl suberimidate (DMS) was purchased from Pierce. The ^{125}I-labeled protein A and the enhanced chemiluminescence Western blotting system were from Amersham.

Rev Expression Plasmids. All Rev derivatives were constructed by using the coding sequences of previously characterized mutants (17–19). For expression in *Escherichia coli*, the coding sequence for each mutant was obtained from the original plasmid by PCR and inserted between the *Nco* I and *Hin*dIII sites of the bacterial expression plasmid pRW76 (8); the resultant clones were verified by DNA sequence analysis.

***E. coli* Expression and Purification of Rev.** Rev expression was induced in *E. coli* XA-90 cells as described (8). Each of the Rev derivatives was expressed to a roughly comparable level and represented ≈5% of total *E. coli* protein (see below and data not shown). For purification of Rev, 3 liters of cells was harvested and lysed in buffer A [20 mM Hepes/200 mM NaCl/20% (vol/vol) glycerol], cell debris was removed by centrifugation, and the supernatant was loaded directly onto a 400-ml DEAE-Sepharose CL-6B column. The 200 mM salt eluate from this column was fractionated on a 2-ml heparin-Sepharose affinity column. Rev protein was removed by 2 M salt, dialyzed against buffer A, and stored at −80°C. As judged by staining with Coomassie blue, Rev was >98% pure and retained sequence-specific RNA binding activity (data not shown).

RESULTS

Rev Binds to RNA as an Oligomer. Previous studies have shown that Rev binds specifically and tightly to the RRE (8–14). To address whether Rev interacts with the RRE as a monomer, or as an oligomer, we determined the sedimentation coefficient of the Rev–RRE complex. *E. coli*-derived Rev was incubated with a ^{32}P-labeled RRE-containing RNA transcript, the reaction mixture was treated with RNase A and T1, and the products were fractionated on a glycerol gradient. Each gradient fraction was analyzed on a native gel to resolve the ^{32}P-labeled Rev–RRE complex from the RNase digestion products. The results in Fig. 1 show that the Rev–RRE complex peaks in fraction 11, corresponding to a Svedberg coefficient of 4.2 S. Assuming that the Rev–RRE complex is globular with an average partial specific volume and degree of hydration, an S value of 4.2 corresponds to a molecular weight of ≈67,000 (20). These results are most consistent with the possibility that four Rev molecules are bound to the RNase-resistant RRE fragment.

Rev Is a Stable Oligomer in the Absence of the RRE. The results described above are consistent with either of two possibilities. First, multiple Rev monomers could bind independently to the RRE. Alternatively, Rev could be a stable oligomer, which binds to the RRE in a single step. To distinguish between these possibilities, we performed chemical cross-linking experiments to analyze oligomerization of Rev in the absence of the RRE.

Rev was purified to near homogeneity from *E. coli* extracts by using a combination of conventional and affinity chromatography (*Materials and Methods*). Purified Rev was incu-

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Abbreviations: RRE, Rev response element; DMS, dimethyl suberimidate.

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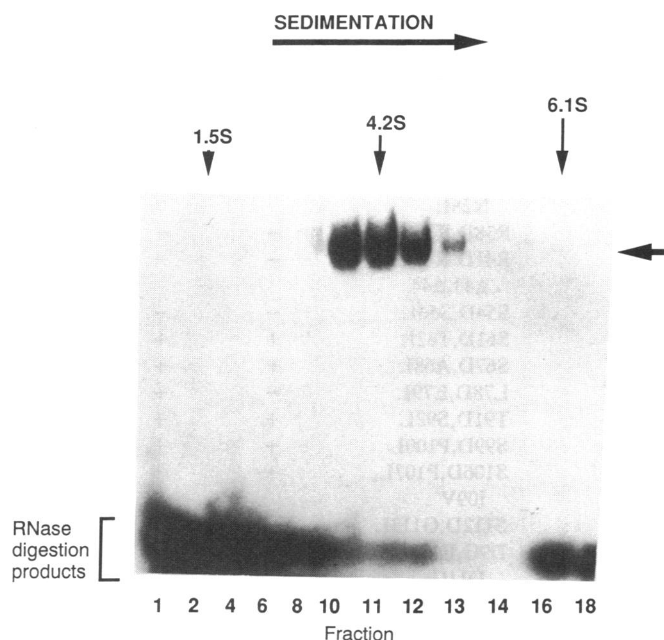

FIG. 1. Sedimentation analysis of the Rev–RRE complex. A ^{32}P-labeled RRE transcript (8) was incubated in a Rev-containing *E. coli* extract and digested with RNases T1 and A; the products were fractionated on a 10–40% glycerol gradient. The gradient was centrifuged in a TLS-55 rotor using a TL-100 centrifuge (Beckmann) for 19 hr at 39,000 × *g* and 4°C. Fifty microliters of each gradient fraction was analyzed on a 6% polyacrylamide/0.5% agarose composite gel. The horizontal arrow indicates the specific Rev–RRE complex. The sedimentation coefficients of molecular weight standards analyzed in parallel are indicated: lysozyme (1.5 S), bovine serum albumin (4.2 S), and T7 RNA polymerase (6.1 S).

bated for various times with either of two bifunctional chemical cross-linkers: glutaraldehyde or DMS. After the cross-linking reaction, the products were fractionated by SDS/PAGE, and Rev was detected by immunoblotting with an anti-Rev antibody (8). Fig. 2 shows that in addition to the monomer, whose denaturing molecular mass is ≈15 kDa, there are three higher molecular mass forms, whose appearance is dependent upon addition of a chemical cross-linker and time of incubation. The denaturing molecular masses of

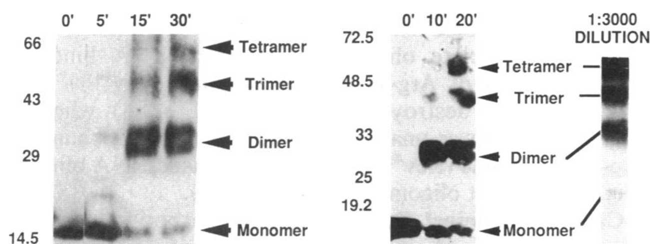

FIG. 2. Chemical cross-linking of purified Rev. (*Left*) Two hundred nanograms of purified Rev was incubated in 300 μl of buffer A containing 0.002% glutaraldehyde at 25°C. Aliquots were removed after 0, 5, 15, and 30 min and analyzed on an SDS/12.5% polyacrylamide gel. Rev was detected by immunoblotting. The higher molecular mass Rev species and their inferred identities are indicated. Positions of molecular size standards (in kDa) are indicated on the left. (*Right*) Seventy-five nanograms of purified Rev in TEAM buffer (0.01 triethanolamine/0.01 $MgCl_2$/0.02 2-mercaptoethanol, pH 9.0) containing DMS at 3 mg/ml was incubated at 25°C. Aliquots were removed after 0, 10, and 20 min; 2 μl of 1 M Tris (pH 7.4) was added; the samples were fractionated on an SDS/12.5% polyacrylamide gel; and Rev was detected by immunoblotting. Alternatively, purified Rev was diluted 3000-fold in TEAM buffer prior to addition of DMS to 3 mg/ml. After a 30-min incubation at 25°C, the samples were processed as described above.

these higher-order forms, approximately 30, 45, and 60 kDa, are consistent with them being a dimer, a trimer, and a tetramer. Identical results were obtained with two other cross-linking reagents, ethylene glycolbis(succinimidylsuccinate) and disuccinimidyl suberate (data not shown).

Although these experiments were performed with purified protein, it remained possible that the higher-order forms represented an interaction between Rev and another contaminating protein or between independent Rev monomers. To rule out these possibilities, we diluted the purified Rev preparation 3000-fold before the addition of the chemical cross-linker. The results indicate that the 30-, 45-, and 60-kDa species were still observed after this substantial dilution. In fact, the amount of the higher-order forms relative to the monomer was significantly increased, presumably because dilution increases the molar ratio of chemical cross-linker to Rev. We conclude that the 30-, 45-, and 60-kDa species represent highly stable protein–protein interactions and do not result from artificially high concentrations of purified Rev. The experiments below show that comparable results were obtained when cross-linking was performed in crude *E. coli* extracts or mammalian cells, rather than with purified protein. Thus, Rev does not stably interact with the many cellular proteins, indicating that the interaction between Rev subunits is highly specific. The specificity of the interaction was confirmed by analysis of Rev mutants (see below). Based upon these combined data, we conclude that Rev exists in solution and binds to RNA as a stable oligomer, most likely a tetramer.

Rev Is an Oligomer in Mammalian Cells. To rule out the unlikely possibility that the *in vitro* results obtained above were unique to *E. coli*-derived Rev, we analyzed Rev oligomerization in transfected mammalian cells. We adapted an *in vivo* cross-linking procedure originally described by Solomon and Varshavsky (21). In brief, COS cells were transfected with a Rev expression plasmid, and after 36 hr the intact cells were treated with glutaraldehyde (Fig. 3 *Lower*). Cell lysates were prepared from the glutaraldehyde-treated cells and fractionated by SDS/PAGE, and Rev was detected by immunoblotting. Fig. 3 *Upper* shows that addition of the chemical cross-linking reagent gave rise to three higher molecular mass Rev species, the sizes of which are expected for a dimer, a trimer, and a tetramer. Thus, both *in vitro* and *in vivo* Rev is a stable oligomer in the absence of the RRE.

Amino Acid Sequences Required for Oligomerization and Sequence-Specific RNA Binding. To delineate the region(s) of Rev involved in oligomerization and sequence-specific RNA binding, we analyzed two series of amino acid substitution mutants whose activity has been previously characterized *in vivo* (17–19). Table 1 describes these mutants and summarizes the results of the experiments presented below. Extracts were prepared from *E. coli* expressing one of these mutants. Oligomerization was assayed by chemical cross-linking (Fig. 4), and sequence-specific RNA binding was measured by an RNase protection/mobility shift assay (8) (Fig. 5). Extracts containing Rev derivatives that were defective in oligomerization or RNA binding were further analyzed in a mixing experiment to rule out the presence of an inhibitor (data not shown).

For discussionary purposes we have divided (somewhat arbitrarily) the Rev protein into three regions: the amino-terminal region (amino acids 1–35), the arginine-rich region (amino acids 36–60), and the carboxyl-terminal region (amino acids 61–116).

Amino-Terminal Region. Previous studies have described substitutions within the amino-terminal portion of Rev, including $Rev^{14-16EED}$, M4 (amino acids 23, 25, and 26), and Rev^{27-29A}, that destroy activity (17, 19). Fig. 4 shows that each of these mutants is oligomerization defective. In contrast, $Rev^{9-11GKG}$ and M3 (amino acids 17 and 18), which have

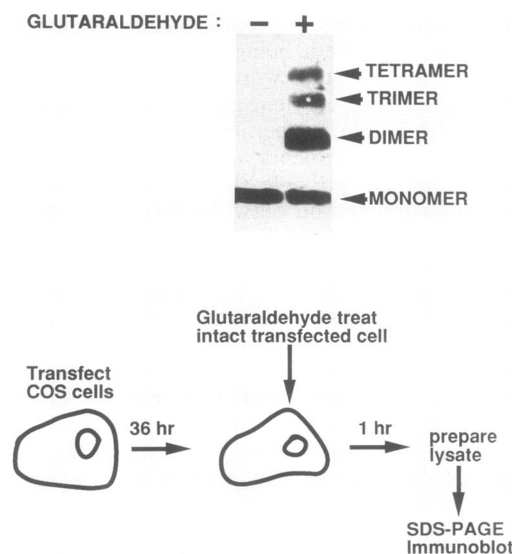

FIG. 3. Rev is a stable oligomer in transfected mammalian cells. (*Upper*) COS cells were transfected with the plasmid pcRev (3) as described by Lin and Green (22). At 36 hr posttransfection, the cells were incubated in Dulbecco's modified Eagle's medium containing 0.3% methanol, 0.01 M NaCl, 0.1 mM EDTA, 5 mM Hepes (pH 7.5), and 0.01% glutaraldehyde. After incubation at 37°C for 10 min, incubation was continued at 4°C for 1–4 hr. Cells were collected at 3000 × *g* for 3 min and resuspended in 500 μl of buffer A containing 0.5% Nonidet P-40. Cells were incubated on ice for 5 min and centrifuged at 3000 × *g* for 3 min to recover nuclei. The nuclei were resuspended in 100 μl of buffer A and treated with 10 units of DNase I and 10 μl of a solution containing RNase A at 5 mg/ml and 100 units of RNase T1. After incubation at 37°C for 15 min, 100 μl of RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) was added, and debris was removed by centrifugation at 13,000 × *g* for 15 min. One hundred microliters was removed and boiled in 50 μl of 9 M urea/4% SDS sample buffer for 5 min; the proteins were resolved on an SDS/12.5% polyacrylamide gel; and Rev was visualized by using the Amersham enhanced chemiluminescence Western blotting detection system. The higher molecular mass Rev species and their inferred identities are indicated. (*Lower*) A schematic diagram of the experimental protocol is shown.

wild-type Rev activity (17, 19), oligomerize normally (Fig. 4). Thus, some residues within the amino-terminal region are involved in oligomerization, and the boundary for oligomerization is at or near residue 14.

Fig. 5 shows that these oligomerization-defective mutants, $Rev^{14-16EED}$, Rev^{27-29A}, and M4, are severely defective for RNA binding. Conversely, $Rev^{9-11GKG}$ and M3 both oligomerize (Fig. 4) and bind RNA (Fig. 5) at wild-type levels.

Arginine-Rich Region. Substitution of multiple residues within the arginine-rich region destroys Rev function (17–19). These multiple substitution mutants include M5 (amino acids 38 and 39), M6 (amino acids 41–44), $Rev^{38,39G}$, Rev^{41-43G}, and $Rev^{48,50G}$. Fig. 4 shows that oligomerization of each of these mutants is severely decreased.

In M7 two serines (amino acids 54 and 56) just past the run of arginines have been changed to glycines, resulting in a loss of Rev function (17). Fig. 4*B* shows that M7 is oligomerization defective.

When only a single residue within the arginine-rich region was substituted, oligomerization was not necessarily disrupted. For example, changing Arg-44 to either tryptophan (Rev^{44W}) or glycine (Rev^{44G}), or changing Trp-45 to arginine (Rev^{45R}), did not affect oligomerization (Fig. 4). Interestingly, when Trp-45 was changed to glycine, oligomerization was abolished.

Fig. 5 shows that all of these oligomerization-defective mutants (M5, M6, $Rev^{38,39G}$, Rev^{41-43G}, $Rev^{48,50G}$, and M7)

Table 1. Description of Rev mutants and summary of results

Mutant	Substitution(s)*	Rev activity	Oligo-merization	RNA binding
M2	S7D,D8L	+	+	+
M3	R17D,L18I	+	+	+
M4	Y23D,S25D, N26L	−	−	−
M5	R38D,R39L	−	−	−
M6	R41D,R42L, Δ43,Δ44	−	−	−
M7	S54D,S56L	−	−	−
M8	S61D,T62L	+	+	+
M9	S67D,A68L	+	+	+
M10†	L78D,E79L	−	+	+
M11	T91D,S92L	+	+	+
M12	S99D,P100L	+	+	+
M13	S106D,P107L, I109V	+	+	+
M14	S112D,G113L	+	+	+
$Rev^{9-11GKG}$	D9G,E10K, D11G	+	+	+
$Rev^{14-16EED}$	K14E,A15E, V16D	−	−	−
Rev^{27-29A}	P27A,P28A, P29A	−	−	−
$Rev^{38,39G}$	R38G,R39G	−	−	−
$Rev^{41,43G}$	R41G,R42G,R43G	−	−	−
Rev^{44W}	R44W	+/−	+	−
Rev^{44G}	R44G	+/−	+	−
Rev^{45R}	W45R	+/−	+	−
Rev^{45G}	W45G	−	−	−
$Rev^{48,50G}$	R48G,R50G	−	−	−

Mutants M3–M14 were originally described by Malim *et al.* (17), and $Rev^{9-11GKG}$–$Rev^{48,50G}$ were described by Hope *et al.* (18, 19). *In vivo* Rev activity was determined by Malim *et al.* (17) and Hope *et al.* (18, 19). +, Wild-type Rev activity; +/−, incomplete Rev activity; −, no detectable or severely decreased Rev activity. Oligomerization and RNA binding activities were determined in this report.

*The amino acid substitutions are designated as follows: the wild-type residue (one-letter code) and its position number are given, followed by the substituted amino acid residue. (For example, S7D designates the replacement of Ser-7 by Asp.)

†Dominant-negative mutant.

were correspondingly defective in sequence-specific RNA binding. The single-amino-acid substitution mutants did, however, dissociate oligomerization from RNA binding. First, mutation of Arg-44 to a tryptophan (Rev^{44W}) or a glycine (Rev^{44G}) destroyed RNA binding (Fig. 5*A*), whereas oligomerization was unaffected (Fig. 4*A*). Second, changing Trp-45 to arginine (Rev^{45R}) severely decreased RNA binding (Fig. 5*A*) but not oligomerization (Fig. 4*A*).

Carboxyl-Terminal Region. Mutants harboring substitutions within the carboxyl-terminal portion of Rev, including M8 (amino acids 61 and 62), M9 (amino acids 67 and 68), and M10 (amino acids 78 and 79) oligomerize normally (Fig. 4*B*; data not shown). M8 and M9 have wild-type activity, whereas M10 lacks Rev function and has a dominant-negative phenotype (17). Previous studies have shown that the carboxyl-terminal 25 amino acids are not required for Rev function (17). Accordingly, mutants M11 (amino acids 91 and 92), M12 (amino acids 99 and 100), M13 (amino acids 106, 107, and 109), and M14 (amino acids 112 and 113) oligomerize normally (data not shown). Based upon these results, we conclude that the carboxyl-terminal portion of Rev is not involved in oligomerization.

Fig. 5*B* shows that all of the mutants in the carboxyl-terminal portion of Rev (M8, M9, M10, M11, M12, M13, and M14) bind RNA normally. Of particular significance is M10,

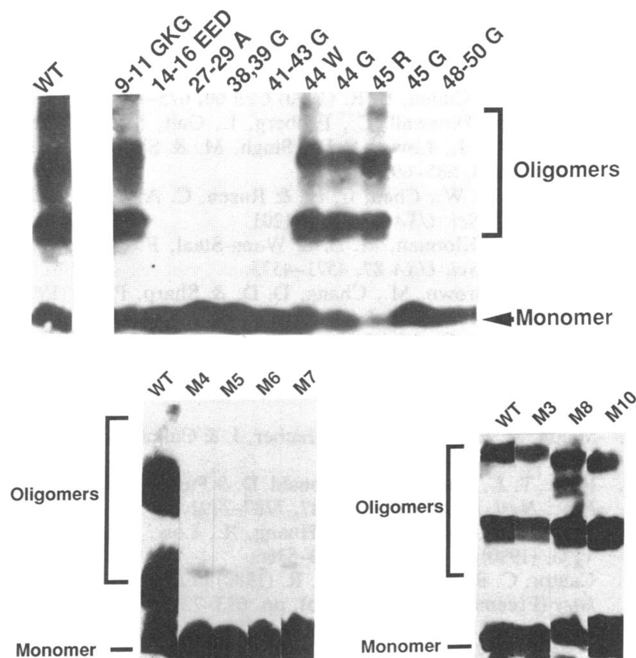

FIG. 4. Analysis of Rev mutants by chemical cross-linking. Twenty microliters of each *E. coli* lysate was incubated at 25°C for 15 min in TEAM buffer containing DMS at 3 mg/ml. After incubation, the samples were centrifuged for 5 min at 13,000 × *g*, and the supernatants were boiled in an SDS-loading buffer and fractionated on an SDS/12.5% polyacrylamide gel. Rev species were detected as described in Fig. 3. Note that because of the high total protein concentration in the *E. coli* lysate, less tetramer was observed than when purified Rev was used (Fig. 2). (*Upper*) Analysis of Rev mutants described by Hope *et al.* (19). (*Lower*) Analysis of Rev mutants described in Malim *et al.* (17). WT, wild type.

which lacks Rev activity and has a dominant-negative phenotype (17).

We summarize these data as follows. First, both oligomerization and RNA binding are dependent upon a region encompassing amino acids 14–60. Second, all mutations that disrupted oligomerization also abolished RNA binding, which strongly suggests that oligomerization is required for RNA binding. Third, at least two residues, Arg-44 and Trp-45, have a direct role in RNA binding.

DISCUSSION

Based upon previous mutagenesis studies (17–19, 23, 24) and our results, we propose that Rev contains at least two functional domains. One domain, the oligomerization/RNA binding region, is required for targeting to the nucleic acid. A second separable domain is required for Rev activity *in vivo* but is dispensable for oligomerization and RNA binding *in vitro*. The boundaries of this latter region have not been defined, but they must encompass amino acids 78 and 79, which are altered in M10 (17). Because the precise mechanism of Rev action is not understood (25), we refer to this latter region as an "effector" domain. The effector domain presumably interacts with cellular factors involved in either mRNA nuclear export and/or pre-mRNA splicing.

Our data indicate that Rev's oligomerization/RNA binding domain extends from approximately residues 14 to 60 and involves amino acids of all classes. Surprisingly, multiple substitutions within the arginine-rich region disrupted oligomerization; one might have predicted that this positively charged region was involved solely in an RNA–protein interaction. The most likely interpretation of these combined results is that oligomerization results from highly specific interactions between Rev subunits.

Every oligomerization-defective mutant also failed to bind RNA *in vitro* and support a complete Rev response *in vivo* (Table 1). The excellent correspondence between oligomerization, RNA binding, and Rev activity argues strongly that the oligomerization characterized here is essential for Rev function. These correlative data also suggest that the RNase protection/mobility shift assay, which we use to quantitate RNA binding *in vitro*, accurately reflects Rev's RNA binding activity *in vivo*. Our results do not exclude the possibility that some mutants, which we categorize as RNA binding defective, can be shown to interact with the RRE in other RNA binding assays. For example, peptides containing a minimal arginine-rich motif can be shown to bind RNA in a mobility shift assay (26). However, we find that such peptides, like oligomerization-defective mutants, do not bind RNA in the RNase protection/mobility shift assay (Fig. 5; unpublished data). Thus, compared to wild-type Rev, such Rev derivatives must bind the RRE at a lower affinity and/or with a faster off rate.

Substitution of at least two amino acids within the arginine-rich motif, Arg-44 and Trp-45, generates Rev mutants that oligomerize but do not bind RNA. Thus, although oligomerization appears to be essential for RNA binding, the two activities can be separated. The most likely explanation of these results is that Arg-44 and Trp-45 directly contact RNA.

Based upon both chemical cross-linking data and sedimentation analysis of the Rev–RRE complex, we estimate that Rev is a stable tetramer. This conclusion is consistent with the behavior of *E. coli*-derived Rev on a gel-filtration column (27) and high-resolution footprinting of Rev on the RRE (14). However, additional information is required to establish definitively the number of subunits in the Rev oligomer. Two other proteins containing the arginine-rich motif, Tat (28) and Gag (29), also appear to be oligomers. Oligomerization may

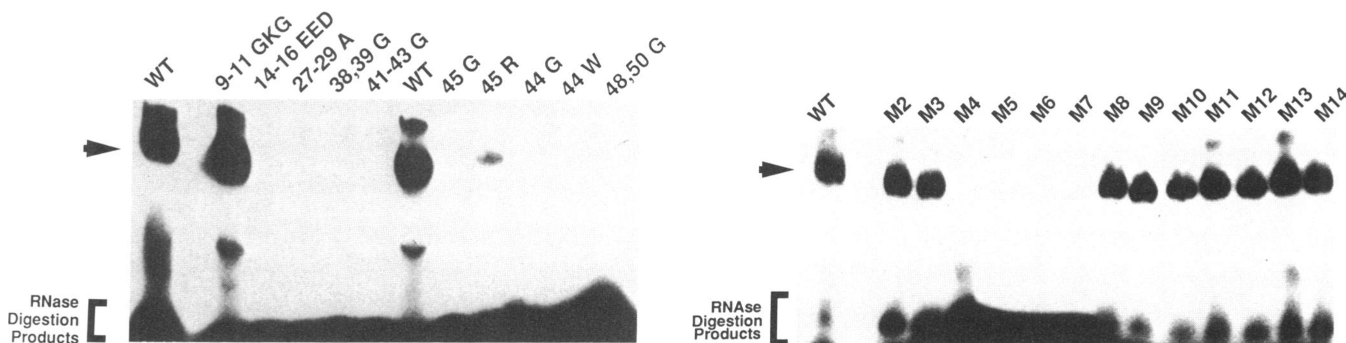

FIG. 5. Sequence-specific RNA binding activity of mutant Rev proteins. Sequence-specific RNA binding of Rev mutants was analyzed by an RNase protection/mobility shift assay using a ^{32}P-labeled RRE substrate (8). The arrow indicates the Rev–RRE complex. (*Left*) Mutants described by Hope *et al.* (18, 19). (*Right*) Mutants described by Malim *et al.* (17). WT, wild type.

thus be a general feature of this class of RNA binding proteins.

Our conclusion that Rev is an oligomer in the absence of the RRE is in agreement with previous *in vitro* experiments of Olsen *et al.* (23). In contrast, Malim and Cullen (30) have recently concluded that Rev is a monomer, which oligomerizes only upon binding to RNA (30). This study further suggested that oligomerization of Rev on RNA was involved in the mechanism by which human immunodeficiency virus is activated from latency (30). Among the many differences between our study and that of Malim and Cullen (30), one striking feature is that in the majority of their experiments Malim and Cullen (30) used a glutathione *S*-transferase (GST)–Rev fusion protein. We have found that comparable GST–Rev fusion proteins are severely defective for RNA binding *in vitro* and Rev function *in vivo* (unpublished data). It seems likely that the differences in activities between Rev and GST–Rev account, at least in part, for this discrepancy.

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