Scanning Mutagenesis of the Arginine-Rich Region of the Human Immunodeficiency Virus Type 1 Rev \textit{trans} Activator

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The structural proteins of human immunodeficiency virus type 1, for example, Gag and Env, are encoded by unspliced and incompletely spliced viral transcripts. The expression of these mRNAs in the cytoplasm, along with their commensurate translation, is absolutely dependent on the virally encoded Rev \textit{trans} activator. Previous studies have demonstrated that Rev binds directly to its substrate mRNAs via an arginine-rich element that also serves as its nuclear localization sequence. In an attempt to define the specific amino acid residues that are important for in vivo activity, we have constructed a series of missense mutations that scan across this region. Our data demonstrate that all eight arginine residues within this element can, individually, be substituted for either leucine or lysine with no apparent loss of function. Importantly, these findings suggest that no single amino acid within the arginine-rich domain of Rev is, by itself, essential for activity and that considerable functional redundancy is therefore likely to exist within this region. Interestingly, one mutant in which a tryptophan had been substituted for a serine failed to accumulate exclusively in the nucleus but still bound RNA in a manner that was indistinguishable from that of the wild-type protein. This observation indicates that features of the arginine-rich region that are additional to those required for RNA binding are important for Rev's correct accumulation in the nucleus.

Human immunodeficiency virus type 1 (HIV-1) utilizes alternative splicing of its full-length ~9-kb primary transcript to generate the array of mRNAs necessary for the balanced expression of all viral proteins (for reviews, refer to references 11 and 33). In particular, the 9-kb and singly spliced ~4-kb mRNAs (which encode Gag, Pol, Env, Vif, Vpr, and Vpu) may be regarded as intron containing, whereas the fully spliced ~2-kb mRNAs (which encode Tat, Rev, and Nef) are, by definition, intron lacking. Importantly, the cytoplasmic expression of the 9- and 4-kb mRNAs is fully dependent on the Rev \textit{trans} activator (7, 15, 16, 18, 20, 26, 27). In the absence of Rev, these intron-containing transcripts are retained in the nucleus and either spliced to completion or subjected to degradation. The \textit{cis}-acting target for Rev is a complex stem-loop RNA element, the Rev response element (RRE), that resides within the \textit{env} open reading frame and is therefore represented in all 9- and 4-kb viral transcripts (17, 27, 36). Rev, a protein that localizes to the nuclei and particularly to the nucleoli of cells during interphase (12, 16, 24, 34), binds directly to the RRE both in vitro and in vivo (13, 39, 43).

Mutational analyses of the HIV-1 Rev protein have demonstrated that there are two distinct domains, both of which are essential for \textit{trans} activation. Toward the center of the more N-terminal domain is a stretch of amino acids (residues 33 to 46 of the 116-amino-acid Rev protein of the HXB-3 isolate) that contains eight arginine residues. This arginine-rich sequence has been shown to serve as Rev's nuclear localization sequence (8, 24, 34) and to mediate the direct binding of Rev to the RRE (4, 25, 31, 39, 44). Moreover, it is this basic stretch of amino acids that confers RNA target specificity on Rev. For example, when the basic domain of the human T-cell leukemia virus type I counterpart of Rev, which is known as Rev, was replaced with the arginine-rich domain of HIV-1 Rev, the resulting chimeric Rev/Rex protein displayed the same RNA target specificity as Rev (4). Also included within the N-terminal domain of Rev are residues that participate in the assembly of Rev into multimeric complexes; these sequences are less well defined but are known to extend away from the arginine-rich region in both directions (25, 31, 44).

The C-terminal domain of HIV-1 Rev is more compact than the N-terminal domain and has been mapped to residues 75 to 93 (19, 21, 24, 28, 29, 41, 42). The critical features of this region are four evenly spaced hydrophobic residues, most commonly leucines, the mutation of which generates nonfunctional proteins that retain wild-type RNA binding and nuclear localization characteristics (14, 24, 25, 31, 39, 44). By analogy with the domain organizations of a number of transcription factors, this region has been operationally defined as either the activation domain or the effector domain (24, 44). Accordingly, it was proposed that this domain is likely to mediate the interaction of Rev with host cell proteins involved in the translocation of RNA to the cytoplasm. Indeed, in a recent study, we identified nuclear eukaryotic initiation factor 5A as a protein that interacts with the activation domain of Rev and mediates \textit{trans} activation (37).

The purpose of this study was to assess the importance of individual amino acids of HIV-1 Rev's arginine-rich domain for \textit{trans} activation in vivo. To our surprise, we found that single missense mutations between positions 33 and 46 are well tolerated with respect to protein function. These data, therefore, suggest that no individual residue in this region is essential for Rev-mediated \textit{trans} activation.

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MATERIALS AND METHODS

Molecular clones. The following plasmids have been described previously. pgTAT encodes the viral tat gene in the configuration in which it resides in the provirus; specifically, the two coding exons of tat are separated by an intron that contains the RRE (27). pcREV encodes a cDNA copy of the rev gene of the HXB-3 isolate (27). pBC12/CMV/IL-2 serves as a negative control plasmid in transfection experiments (10). pSLLIB/CAT contains an HIV-1 long terminal repeat promoter cassette in which the apical loop and the most 3’ pyrimidine of the bulge of the trans-activation response element have been replaced with the RRE-derived high-affinity binding site for Rev; this drives expression of the bacterial chloramphenicol acetyltransferase (CAT) gene (39). pcTat/Rev expresses a chimeric protein in which the C terminus of Tat is fused to the N terminus of Rev; this protein retains both Tat and Rev activity in vivo (39). pCH110 (Pharmacia Biotech Inc., Piscataway, N.J.) expresses β-galactosidase under the control of the simian virus 40 early promoter and serves as an internal control for transfection efficiency. pGST-Rev is an Escherichia coli vector that expresses Rev fused to the C terminus of glutathione-S-transferase (GST) (25). pGEM/RRE is an in vitro transcription vector that contains the RRE (25). Oligonucleotide-directed mutagenesis with a bacteriophage M13 mutagenesis system (Amersham Corp., Arlington, Ill.) was used to introduce targeted nucleotide substitutions encoding single amino acid alterations into the rev gene of pcREV. All mutations introduced were confirmed by DNA sequencing (Sequenase 2.0; United States Biochemicals, Cleveland, Ohio) and by predicting the wild-type amino acid, amino acid position, and introduced residue (Table 1); for example, RevG33A represents a alanine for glycine substitution at position 33. The variants of pGST-Rev and pcTat/Rev that possess mutated rev genes were generated by straightforward exchange of the wild-type gene following PCR (30)-mediated amplification of the mutant gene.

Cell culture, transfections, and CAT assays. The cell lines COS and HeLa were maintained and transfected with either DEAE-dextran and chloroquine or calcium phosphate as previously described (39). For analysis of Rev-mediated trans activation, COS cells (2.5 × 10⁵) were cotransfected with 200 ng of pgTAT in combination with 100 ng of either pBC12/CMV/IL-2 (negative control), pcREV (positive control), or mutant Rev expression vector. Confirmation of Rev protein expression was performed by transfecting 2.5 × 10⁵ COS cells with 500 ng of either pBC12/CMV/IL-2, pcREV, or mutant Rev expression vector. To determine the subcellular localization of Rev proteins, 2.5 × 10⁵ COS cells were transfected with 500 ng of expression plasmid by using Lab-Tek two-chamber slides (Nunc, Inc., Naperville, Ill.). For analysis of the in vivo binding characteristics of Tat-Rev fusion proteins, 2.5 × 10⁵ HeLa cells were either mock transfected or transfected with 2.5 μg of pSLLIB/CAT, 2.5 μg of either pBC12/CMV/IL-2 (negative control), pcREV (positive control), or mutant Tat/Rev expression vector, and 2.5 μg of pCH110 (7.5 μg of total plasmid DNA); at ~48 h, cell lysates were prepared and the levels of CAT and β-galactosidase activity were determined as described previously (39).

Antisera, radioimmunoprecipitation, and indirect immunofluorescence. Polyclonal anti-Rev-specific antisera were raised in New Zealand White rabbits by using standard procedures and purified recombinant Rev protein (13). Monoclonal anti-Rev antibodies that specifically recognize the C terminus of Rev have been described previously (3). The nature of Tat protein expression in pgTAT-transfected cells (86-amino-acid form versus 72-amino-acid form) was evaluated by metabolic labeling at ~48 h posttransfection and immunoprecipitation before the resolution of Tat proteins on sodium dodecyl sulfate-polyacrylamide gels (27). Radioimmunoprecipitation of transiently transfected COS cell cultures was also used to monitor the expression of rev mutant genes. Indirect immunofluorescence was performed on transfected and paraformaldehyde-fixed cultures of COS cells with a mouse monoclonal anti-Rev antibody at a 1:1,000 dilution followed by a Texas red-conjugated anti-mouse antibody raised in goats at a 1:50 dilution (24).

Purification of GST-Rev fusion proteins and in vitro RRE binding. GST-Rev fusion proteins were expressed in E. coli BL21 and initially purified from crude lysates by affinity chromatography with glutathione-Sepharose 4B according to the manufacturer’s specifications (Pharmacia Biotech Inc.). Eluted fusion proteins were further subjected to size exclusion chromatography in phosphate-buffered saline with Superose 12 (Pharmacia Biotech Inc.). Rev-containing fractions were identified by Western immunoblot analysis, pooled, concentrated by ultrafiltration with a PM10 filter device (Amicon Inc., Beverly, Mass.), and stored at −70°C. Final protein concentrations were determined by the method of Bradford (5). RNA binding assays with a 252-nucleotide radiolabeled RRE probe were performed as previously described (25) except that RNA-protein complexes were resolved on 6% polyacrylamide gels (Mini Protein II; Bio-Rad Laboratories, Hercules, Calif.).

RESULTS

In contrast to the C-terminal activation domain, the arginine-rich domain of Rev (here defined as residues 33 to 46) has not been subjected to detailed single amino acid substitution mutagenesis. In particular, many of the mutations that have been described for this region contain either deletions or multiple substitutions (20, 21, 24, 31). There has, however, been one report in which a set of scanning alanine substitutions was introduced across this domain (38). Although this study concluded that the arginines at positions 35, 38, 39, and 44, as well as the threonine at position 34 and the asparagine at position 40, were all important for sequence-specific RRE binding (and by extrapolation, for protein function), these mutations were analyzed only in the context of synthetic peptides. Interestingly, these experiments did indicate that the arginine-rich domain adopts an alpha-helical conformation in solution and that increasing helicity correlates with enhanced RRE binding.

Scanning mutagenesis of the arginine-rich domain of Rev. The aim of this study was to determine which amino acids of the arginine-rich domain are important for trans activation in vivo. To accomplish this, we introduced a series of scanning missense mutations between residues 33 and 46 of the rev gene of pcREV (Table 1). Given that we wanted to identify amino acids that serve a supplementary specific, as opposed to a structural, role in Rev function, we chose to introduce amino acids that are predicted to be favorable toward alpha-helix formation (32, 35). In particular, all eight arginines were replaced with either lysine, which maintains the positive charge, or leucine, which removes the positive charge. The remaining residues were all replaced with amino acids with side chains that are similar as well as dissimilar in character.

We have previously described a transfection-based assay in which expression of the viral Tat protein is used to monitor Rev function (27). In such assays, the genomic tat expression vector, pgTAT, is cotransfected into COS cells along with the vector in question. Because HIV-1 pre-mRNAs are spliced...
TABLE 1. Description and trans-activation phenotypes of HIV-1 Rev mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Arginine-rich region (amino acids 33 to 46)</th>
<th>trans activation&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev (WT)</td>
<td>G T R Q A R R N R R R R W</td>
<td>+</td>
</tr>
<tr>
<td>RevG33A</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>RevG33D</td>
<td>D</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>+</td>
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<td>K</td>
<td>+</td>
</tr>
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<td>L</td>
<td>+</td>
</tr>
<tr>
<td>RevQ36N</td>
<td>N</td>
<td>+</td>
</tr>
<tr>
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<tr>
<td>RevR46L</td>
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<sup>a</sup> trans-activation ability was determined by cotransfection into COS cells with pgTAT and immunoprecipitation with an anti-Tat antiserum. A functional Rev protein was defined as that which induced expression of the truncated 72-amino-acid form of the Tat protein; no partial phenotypes were reproducibly observed. The results of three independent experiments are represented. +, Rev function detected; --, no Rev activity.

*WT, wild type.

Inefficiently in primate cells (7, 28), relatively high levels of both unspliced and spliced tat-specific transcripts are present in the nuclei of pgTAT-transfected cells. In the absence of functional Rev, only spliced transcripts, which encode an 86-amino-acid Tat protein, are expressed in the cytoplasm. In contrast, when Rev is present, unspliced transcripts are retrieved in the cytoplasm. Because these intron-containing transcripts encode a Tat protein of 72 amino acids, this shortened Tat can be used to monitor Rev activity. Therefore, we cotransfected COS cells with pgTAT and either a negative control vector, pcREV, or each of the missense mutant vectors. Rev function was then assessed as described above by determining the pattern of Tat expression in each culture by radioimmunoprecipitation (data not shown). Remarkably, as summarized in Table 1, the majority of mutants were able to induce expression of the 72-amino-acid form of Tat and can therefore be regarded as competent for trans activation. Two mutants, namely RevN40D and RevW45S, failed to trans activate; this was not due to inadequate expression in transfected cells as radioimmunoprecipitation with a polyclonal anti-Rev antiserum revealed that both of these inactive proteins as well as all the functional mutants were expressed at levels comparable to that of wild-type Rev (Fig. 1; for RevW45S and RevN40D, refer to Fig. 1B, lane 15, and C, lane 3, respectively).

Subcellular localization and RRE binding phenotypes of RevN40D and RevW45S. To date, all reported analyses of Rev mutants carrying missense or deletion mutations in the arginine-rich domain have indicated that this region is responsible for Rev’s localization to the nucleus (8, 24, 34) as well as for mediating its direct interaction with the RRE (4, 25, 31, 39, 44). Indeed, no mutations that disrupt one of these essential activities but not the other have been identified in this domain. Such findings have led to the supposition that the sequence requirements for Rev’s accumulation in the nucleus and those
for its binding to the RRE may be the same. We, therefore, wanted to establish whether this correlation also holds true for the two nonfunctional mutants identified here, RevN40D and RevW45S.

To determine the subcellular localization of these two mutant proteins, COS cells were transfected with the relevant expression vectors or pcREV and subjected to indirect immunofluorescence analyses with an anti-Rev monoclonal antibody (Fig. 2). Consistent with previous observations (8, 12, 16, 24, 34), the wild-type protein localized to the nuclei, and primarily the nucleoli, of transfected cells during interphase (Fig. 2A and B). In contrast, neither of the Rev mutants displayed this characteristic nuclear/nucleolar pattern of staining. RevN40D was detected predominantly in the cytoplasm and, in fact, appeared to be excluded from the nucleus (Fig. 2C and D); RevW45S did not accumulate in either the nucleus or the cytoplasm and therefore appeared to be distributed evenly throughout the cell (Fig. 2E and F).

We have previously described an in vitro RNA binding assay in which the ability of bacterially expressed and purified Rev protein to interact with the RRE is assessed by gel retardation (25). As a first step toward determining the RRE binding characteristics of RevN40D and RevW45S, we purified both proteins in the context of fusons to GST. These proteins as well as wild-type Rev, both as a nonfusion protein and as a fusion to GST, were then analyzed for their RNA binding capabilities with a 252-nucleotide RRE probe (Fig. 3). As expected, adding increasing amounts of either Rev or GST-Rev to the binding reaction resulted in increasing incorporation of the RRE into RNA-protein complexes (Fig. 3A). The clear resolution of the retarded probe into a ladder of multiple distinct bands has been shown to be due to the cooperative binding of multiple Rev molecules to a single RRE (9, 14, 25). Interestingly, when the binding phenotypes of GST-RevN40D and GST-RevW45S were examined, quite different results were obtained. GST-RevN40D was unable to bind to the RRE even at the highest concentration of input protein (Fig. 3B, lanes 2 to 5), whereas GST-RevW45S appeared to bind to and multimerize on the RRE in a manner that was comparable to that of GST-Rev (Fig. 3B, lanes 6 to 9).

Previous mutational analyses of the RRE combined with a variety of in vitro binding studies have defined a region known as stem-loop IIB (SLIIB) as the high-affinity binding site for Rev (2, 22, 40). More recently, the identification of this site has been exploited for the development of an in vivo assay for analyzing the interaction of Rev with RNA (Fig. 4A) (39). The reporter plasmid in this assay system, pSLIIB/CAT, contains the CAT gene under the transcriptional control of an HIV-1 long terminal repeat in which the pyrimidine at the 3' boundary of the bulge and the apical loop of the trans-activation response element have both been replaced by SLIIB. Because Tat by itself cannot function through this hybrid RNA element, Tat-dependent trans activation is achieved only when Tat is expressed in the context of a fusion protein with Rev. Since the induction of CAT activity is therefore dependent on Rev's interaction with SLIIB, this assay serves as a sensitive in vivo RNA binding assay for Rev.

To evaluate the SLIIB binding characteristics of RevN40D and RevW45S in vivo, both mutants as well as the functional missense mutant RevN40Q were used to create three variants of pcTat/Rev. These four vectors and a negative control plasmid were each cotransfected into HeLa cells with pSLIIB/CAT, and the resulting extents of trans activation were determined by measuring CAT activity ~48 h later (Fig. 4B). As noted previously, pcTat/Rev is an effective inducer (~85-fold trans activation) of CAT expression in this system (39). With respect to the fusions containing mutated Rev moieties, Tat/N40D was unable to activate transcription, Tat/W45S trans activated to the same extent as wild-type Tat/Rev, and Tat/N40Q exhibited an intermediate phenotype. Importantly, these
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findings are in excellent agreement with the results of our earlier in vitro experiments (Fig. 3) and confirm not only that RevN40D fails to bind to the RRE but that RevW45S can bind to the RRE as efficiently as the wild-type Rev protein.

**DISCUSSION**

This study was undertaken in an effort to identify specific amino acids within the arginine-rich domain of HIV-1 Rev that are required for trans activation in vivo. As noted earlier, most mutations that have been described for this critical domain affected multiple residues and were likely, therefore, to have induced severe structural perturbations (20, 21, 24, 31). Given that this domain forms an alpha-helix in solution (1, 38), a more systematic approach to mutagenesis appears to be warranted. Therefore, we used oligonucleotide-directed mutagenesis to generate a comprehensive set of scanning single amino acid missense mutations between positions 33 and 46 of the HIV-1 Rev protein (Table 1). In particular, we elected to introduce substitutions that would not be expected to interfere with the alpha-helical conformation of this region (32, 35). The subsequent scoring of the trans-activation phenotypes of these mutant proteins suggested that all but two of the mutants retained biological activity and that mutations at all positions within this region could be accommodated with respect to Rev function. Here, we discuss (i) the two novel inactivating mutations that we have identified and (ii) our discovery that the arginine-rich domain of Rev possesses a remarkably high degree of tolerance for missense mutation.

In an effort to more fully understand the nature of the defects in the two biologically inactive mutants, both proteins were subjected to a number of additional assays. The RevN40D mutant, in which the asparagine at position 40 had been changed to a residue that carries a negative charge (in this case, aspartic acid), neither localized to the nucleus (Fig. 2C and D) nor bound to the RRE (Fig. 3B and 4B). We, therefore, consider it likely that this particular mutation, conceivably by favoring the formation of salt bridges, leads to dramatic alterations in the arginine-rich domain that affect multiple aspects of Rev function. The substitution of serine for tryptophan at position 45 in the RevW45S mutant yielded a protein with a rather more informative and interesting phenotype. Although this mutant was biologically nonfunctional, its binding to the RRE both in vitro (Fig. 3B) and in vivo (Fig. 4B) appeared to be indistinguishable from that of wild-type Rev. This finding was unanticipated since all reported inactive mutants of Rev that carry disruptions in the N-terminal domain are either unable to bind to the RRE in vitro or, if they

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**FIG. 3.** In vitro RRE binding analyses of wild-type and mutant Rev proteins. Recombinant Rev (A) and mutant Rev (B) proteins were expressed and purified from E. coli and used in gel retardation studies in conjunction with a radiolabeled RRE probe. The position of unbound RRE is visualized in lane 1 of each gel. (A) Comparison of RRE binding with increasing quantities of the 116-amino-acid form of Rev (lanes 2 to 5) or the GST-Rev fusion protein (lanes 6 to 9). (B) RRE binding characteristics of the GST-RevN40D (lanes 2 to 5) and GST-RevW45S (lanes 6 to 9) mutant proteins.

**FIG. 4.** In vivo RRE binding analyses of wild-type and mutant Rev proteins. (A) Diagrammatic representation of the pSLIB/CAT reporter system. The TAR/SLIB chimeric response element is shown as a solid ball and stick (see the text for further details). (B) Transcriptional trans activation mediated by Tat/Rev fusion proteins. In terms of the absolute levels of transacetylation observed, one typical experiment yielded 627 cpm in the mock-transfected culture, 3,625 cpm in the culture cotransfected with pSLIB/CAT and pcTat/Rev, and 663 cpm in the culture transfected with pSLIB/CAT and the negative control vector, pB12/CMV/IL-2. All CAT values were adjusted for transfection efficiency by determining the level of β-galactosidase in each culture. Data are averages from three separate experiments.
do bind, are unable to multimerize thereafter (25, 31, 44). Thus, for the first time, we have identified an inactivating mutation in a region of Rev that is distinct from the C-terminal activation domain and does not, as far as we can discern, affect binding to the RRE in either a quantitative or qualitative way. On the basis of our immunofluorescence analysis (Fig. 2E and F), we propose that the lack of trans activation observed with RevW4SS is likely to be caused by its inability to localize efficiently and appropriately to the nucleus. It therefore appears that the requirements for the accumulation of Rev in the nucleus and those for its recognition of the RRE, although overlapping, are not identical.

The ability of the arginine-rich domain of Rev to withstand single amino acid mutations was surprising in light of an earlier study in which the exchange of the threonine at position 34, the asparagine at position 40, and any of the arginines at positions 35, 38, 39, or 44 for alanine disrupted binding to the RRE in vitro (38). Although alanine, like lysine and leucine, should maintain alpha-helicity, that study differed from ours in that peptide mimics containing merely 17 residues of Rev sequence were used for binding assays and no attempt was made to correlate the findings with biological activity. In contrast, the results presented in this report used in vivo trans activation to determine the impact of mutations in this domain on Rev activity. Of particular note, we have demonstrated that each of the aforementioned arginine residues can be exchanged for leucine, mutations that therefore eliminate a positively charged residue, with no resulting inhibition of function (Table 1). Because Rev must bind to the RRE for trans activation to take place, we infer that all mutants that are able to function must, by definition, be able to bind specifically to the RRE.

What then could be the basis for the striking differences between the protein sequence requirements for RRE binding observed here and those described previously for synthetic peptides? Because Rev-mediated trans activation depends not only on binding to the RRE (4, 25, 31, 44) but also on contacts between Rev monomers (intramolecular interactions or multimerization), contacts between various subdomains of individual Rev monomers (intramolecular interactions) (1), and interaction(s) with a host cell protein(s) (28, 37), we hypothesize that the amino acid requirements for RNA binding may be less constrained in the context of the full-length protein. Specifically, we suggest that disruptions in one region (in this case, the arginine-rich domain) may be tolerated in the context of the full-length Rev protein because of the variety of compensatory protein-protein interactions that can occur in vivo. In contrast, we propose that such perturbations may be less tolerated in the context of peptides of 17 amino acids because of the inability of these short sequences to participate in such compensatory interactions. For example, all these peptides lack the sequences that flank both sides of the arginine-rich domain and have been shown to be involved in the multimerization of Rev (25, 31, 44).

Interestingly, the tolerance of Rev's arginine-rich domain to single amino acid substitution mutagenesis is highly reminiscent of observations made with the other RNA-binding regulatory protein of HIV-1, Tat (6). In that case, the exchange of individual residues in Tat's arginine-rich domain for alanine also indicated that no single amino acid in this region was critical for the trans activation of viral transcription by Tat. Whether the arginine-rich RNA-binding domains of other proteins (for examples, see reference 23) also turn out to harbor such apparent functional redundancy remains an open question.

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