Mutational Analysis of the Human Immunodeficiency Virus Type 1 Rev Transactivator: Essential Residues Near the Amino Terminus

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The expression of certain mRNAs from human immunodeficiency virus type 1 (HIV-1) is controlled by the viral transactivator Rev, a nucleolar protein that binds a *cis*-acting element in these mRNAs. Rev is encoded by two viral exons that specify amino acids 1 to 26 and 27 to 116, respectively. Earlier studies have mapped essential regions of the protein that are encoded in the second exon. By further mutational analysis of Rev, we have now identified a novel locus encoded by the first exon that also is essential for transactivation *in vivo*. Defined by mutations at residues 14 to 20, this locus coincides with a cluster of positively charged and nonpolar amino acids that is conserved in Rev proteins of all known primate immunodeficiency viruses. Rev proteins that contained mutations at this site were defective in both nuclear localization and transactivation and did not function as *trans*-dominant inhibitors of wild-type Rev. Fusion of these mutants to a heterologous nuclear protein complemented the defect in localization but did not restore biological activity. Our findings suggest that this N-terminal locus may play a direct role in transactivation, perhaps contributing to essential protein-protein interactions or forming part of the RNA-binding domain of Rev.

The *rev* gene product of human immunodeficiency virus type 1 (HIV-1) is a nuclear RNA-binding protein that is essential for HIV-1 replication (3, 5, 7, 14, 27, 31, 32, 34). Localized predominantly in nucleoli (4), Rev protein acts posttranscriptionally to transactivate the expression of incompletely spliced mRNAs that code for structural proteins of the virus. These mRNAs are expressed constitutively in the nuclei of infected cells but can accumulate in the cytoplasm (and are therefore translated) only in cells that express functional Rev (6, 8, 19, 26). This transactivation is sequence specific and is thought to require binding of Rev to an array of RNA hairpin loops, termed the Rev response element (RRE), within the HIV-1 transcripts (5, 13, 20, 34).

Rev is encoded by two viral exons specifying amino acids 1 to 26 and 27 to 116, respectively. Previous studies (18) have distinguished two regions of the protein that are essential for its function *in vivo*. The first of these has been mapped by mutagenesis to amino acids 25 to 50 (18, 23) and so encompasses a prominent arginine-rich tract at residues 35 to 50; this region contains the signals that target Rev to nuclei and nucleoli (18, 23; T. J. Hope, X. Huang, D. McDonald, and T. G. Parslow, *Proc. Natl. Acad. Sci. USA*, in press) and may also participate in binding of the RRE (16). A second region near amino acid 80 is also required for transactivation but appears to have no role in localization of the protein (18). Point mutations outside these two regions have generally been found to have little or no effect on Rev activity. Some evidence suggests, however, that additional sequences near the N terminus might also contribute to function. Malim et al. (18) have observed, for example, that deletion of amino acids 5 to 17 abolishes transactivation, and exon-shuffling experiments suggest that residues within the first 26 amino acids of Rev may be a major determinant of its specificity for the RRE (17). We therefore undertook a further mutational analysis of Rev to identify features at the N terminus that are essential for biological activity.

MATERIALS AND METHODS

Plasmids. The structures of pRSV-Rev, pRSV-Rev/GR, and the Rev-inducible chloramphenicol acetyltransferase (CAT) expression vector pDM128 have been described previously (Hope et al., in press). The Rous sarcoma virus promoter in pRSV-Rev was replaced by the herpesvirus thymidine kinase promoter (bases –109 to +55) to produce ptk-Rev. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel (15), and mutations were confirmed by DNA sequence analysis.

Transfection and CAT assay. COS7 or CV1 cells were transfected by the calcium phosphate method (1). For the CAT assay, each 10-cm plate received 0.25 µg of the β-galactosidase expression vector pCH110 (21) along with the indicated test plasmids and sufficient pUC19 to maintain total DNA input at 10 µg. Cells were harvested 32 to 40 h after transfection and then lysed by three freeze-thaw cycles in 0.2 ml of 0.25 M Tris, pH 7.5. Samples of lysate were assayed for β-galactosidase activity by a colorimetric assay, and volumes that contained equal activity were then assayed for CAT enzyme as described before (10).

Fluorescence immunocytochemistry. COS7 cells growing on glass cover slips were transfected with derivatives of pRSV-Rev or pRSV-Rev/GR (10 µg per 3-cm plate). At 40 h after transfection, the cells were briefly washed twice in phosphate-buffered saline (PBS) and once in ice-cold methanol, fixed in methanol at –20°C for 20 min, and air-dried for 1 h. After rehydration for 15 min, the specimens were incubated for 2 h in the primary antiserum, washed for 15 min, incubated for 1 h in a 1:50 dilution of rhodamine-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim), and then washed again for 15 min, all in PBS at room temperature. Use of the primary GR antiserum has been described (Hope et al., in press). The primary Rev antiserum was a mixture of two rabbit antipeptide sera (each at 1:800 dilution) directed against residues 1 to 20 and 27 to 50, respectively (4), along with a third rabbit serum (a gift from M.-L. Hammar skjöld and D. Rekosh; used at 1:1,500

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dilution) raised against an *Escherichia coli* fusion protein (29) that contained the C-terminal half of Rev.

Western immunoblot analysis. COS7 cells transfected 40 h previously with the indicated plasmids (20 μ g per 10-cm plate) were washed and then suspended in 75 μ l of 100 mM Tris (pH 7.5)–50 mM NaCl–10% glycerol–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–1 mg each of leupeptin, aprotinin, and pepstatin per ml. Cells were lysed by three cycles of freeze-thawing, and samples containing equivalent amounts of total protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose by electroblotting. The membranes were then preincubated for 15 min, incubated for 1 h with the mixed primary Rev antisera described above, briefly washed three times, incubated for 1 h with 5 μ Ci of 125 I-labeled staphylococcal protein A (Amersham), and again washed three times, all at room temperature in PBS containing 2.5% (wt/vol) nonfat dry milk and 0.1% Tween-20.

RESULTS

We have previously (Hope et al., in press) described a cotransfection assay for Rev activity based on a reporter plasmid (pDM128) derived from the 3' half of HIV-1 (Fig. 1A). Transcripts arising from pDM128 encode no functional HIV-1 proteins but contain a single intron that includes both the RRE and the bacterial CAT coding sequence. Because the intron is normally excised during RNA splicing, CV1 cells express only traces of CAT enzyme activity when transiently transfected with pDM128 alone. Cotransfection with the Rev expression vector pRSV-Rev (Hope et al., in press), however, permits unspliced transcripts of pDM128 to enter the cytoplasm and so increases CAT expression 75- to 100-fold relative to an internal control plasmid.

With pRSV-Rev as a template for oligonucleotide-directed mutagenesis, we prepared a series of plasmids that encoded mutant forms of Rev. The mutations included a few deletions and frameshifts as well as individual and clustered missense mutations at selected sites throughout the protein. Each of these mutants was then tested for its ability to induce CAT expression from pDM128 in the cotransfection assay. As summarized in Fig. 1B, we found that various mutations involving amino acids 27 to 50, as well as three different mutations in the C-terminal half of the protein, completely abolished transactivation, whereas mutations in the intervening region (residues 53 to 66) had comparatively little effect. The properties of these mutants were consistent with earlier findings and will not be further elaborated here. In agreement with previous studies (18), we also observed that deletion of N-terminal residues 4 to 26 profoundly inhibited Rev function, suggesting that this region might contain elements required for transactivation.

The Rev protein sequence encoded by pRSV-Rev is that of HIV-1 strain BRU (33); in this sequence, positions 7 to 11 are occupied by a cluster of acidic amino acids that includes one of the two phosphoserines in Rev (18), and positively charged residues are found at positions 4, 14, 17, and 20 (Fig. 2A). A computerized secondary-structure prediction, done by the method of Finer-Moore and Stroud (9), indicated that the N-terminal region of Rev (approximately amino acids 8 to 18) has a very strong propensity to form an α -helix (data not shown).

With these features in mind, we constructed and tested six additional derivatives of pRSV-Rev (designated MA1 through MA6) that contained missense mutations in the

N-terminal region. As illustrated in Fig. 2A, most of these mutations had relatively minor effects on Rev function. Mutations that altered the charge of the acidic region (MA1) or introduced helix-breaking amino acids at position 11 (MA2 and MA3) produced only a partial loss of activity, and replacement of tyrosine by serine at position 23 (MA6) had little or no effect. In striking contrast, however, two mutations (MA4 and MA5) that introduced acidic residues at positions 14 to 16 or 18 to 20, respectively, completely inactivated the protein.

Western blot analysis was then performed to determine whether the effect of these N-terminal mutations resulted from decreased protein stability. When lysates of transfected COS7 cells were analyzed with Rev-specific antisera, each plasmid was found to yield a single predominant immunoreactive protein (Fig. 2C). The mutant proteins were expressed at steady-state levels comparable to that of the wild type and were generally of the expected molecular weights. One interesting exception was MA4, which migrated somewhat more slowly than the other proteins in the denaturing gel, suggesting possible alteration of an unknown posttranslational modification of Rev.

The subcellular distribution of the mutant proteins was analyzed by fluorescence immunocytochemistry in situ. When COS7 cells that had been transfected with wild-type pRSV-Rev were stained with Rev-specific antisera (Fig. 3), immunoreactive protein was found primarily in association with nucleoli, as reported previously (4). This wild-type pattern of localization was also observed for the partially functional mutants MA1, MA2, MA3, and MA6 (data not shown). In contrast, the inactive mutants MA4 and MA5 were each expressed diffusely throughout both nucleus and cytoplasm (Fig. 3). Thus, the inability of MA4 and MA5 to mediate transactivation is associated with a reduced accumulation of the proteins in nuclei of the transfected cells.

We then asked whether the function of these mutants could be restored by correcting the defect in nuclear translocation. We have reported previously (Hope et al., in press) that translocation-defective Rev mutants can be transported efficiently into the nucleus by fusion with a portion of the rat glucocorticoid receptor (GR). Although the GR moiety ensures nuclear translocation of such fusion proteins when steroid is present, signals in the Rev moiety remain essential for nucleolar localization and for transactivation. To test for such signals in the N terminus, we introduced selected mutations into the expression vector pRSV-Rev/GR, which codes for the Rev/GR fusion protein (Fig. 4A). The resulting plasmids were transfected into CV1 cells growing in the presence of a steroid agonist. By Western blotting, each of the mutants was found to be strongly expressed in the transfected cells (Fig. 4B). Every mutant tested (Δ 4-26, MA1, MA4, and MA5) also exhibited a wild-type pattern of localization in hormone-treated cells (e.g., Fig. 4C), implying that these mutations did not inactivate the nucleolar targeting signals in Rev. Unlike the wild-type and MA1 fusion proteins, however, proteins that contained the MA4 and MA5 mutations failed to transactivate CAT expression from pDM128 (Fig. 4D). The latter mutations can therefore abolish transactivation even in the absence of any demonstrable effect on protein localization.

Malim et al. (18) have demonstrated that certain inactive mutant Rev proteins can block the activity of the wild-type protein in *trans*. This dominant-negative phenotype has been observed only for mutations in the C-terminal half of Rev; mutations in the arginine-rich tract have yielded only recessive-negative proteins. To determine whether MA4 or MA5

FIG. 1. Functional analysis of mutant HIV-1 Rev proteins in a transient transfection assay. (A) Derivation of the Rev-dependent reporter plasmid pDM128. Sequences from the 3' half (residues 5863 to 9722) of the genome of HIV-1 strain SF2 (27) are transcribed under the control of the SV40 promoter and enhancer. Details of the construction have been reported (Hope et al., in press). Residual coding regions of the viral *rev*, *env*, and *nef* genes in this construct were made nonfunctional by a 515-bp deletion near the 3' end, by site-directed mutagenesis of the *rev* and *env* initiation codons, and by insertion of a promoterless *cat* gene into the *env* coding region. Locations of the unique splice donor (SD) and acceptor (SA) sites and of the RRE are indicated. LTR, Long terminal repeat. (B) Structure and functional characterization of Rev mutants. Mutations were introduced into the coding region of pRSV-Rev (Hope et al., in press) by oligonucleotide-directed mutagenesis (15). Hatching denotes the location of the arginine-rich tract in the wild-type protein; vertical bars indicate sites of missense mutations. In-frame deletions of indicated codons are designated with Δ; fs58 is a frameshift mutation at codon 58 (Hope et al., in press) that adds a short C-terminal missense sequence (■). Naming of missense mutants indicates the native and mutant residues (in single-letter code) at the affected positions; thus, Y 23 S refers to a substitution of serine for tyrosine at position 23. LE 78,79 DL is identical to the mutant designated M10 in reference 18 and in Fig. 5. The autoradiogram depicts results of CAT enzyme assays of extracts from CV1 cells that had been cotransfected with 0.25 μg of pCH110, 0.5 μg of pDM128, and 1 μg of wild-type or mutant pRSV-Rev 36 h previously; these data are from a single representative experiment. Sham, pUC118 and pCH110 only; None, no pRSV-Rev. Summarized at right are the relative activities of mutants compared with wild-type Rev, based on two or more tests of each construct: +++, 50 to 100%; ++, 25 to 50%; +, 5 to 25%; -, 0 to 5%. All CAT assays in this and other figures were normalized to expression of β-galactosidase activity from pCH110 as an internal control (21).

FIG. 2. Mutations near the N terminus can functionally inactivate Rev. (A) Structures of wild-type and N-terminal mutant forms of pRSV-Rev. This plasmid uses the Rous sarcoma virus (RSV) promoter and HIV-1 polyadenylation signals to transcribe *rev* cDNA sequences derived from HIV-1 strain BRU (33). Hatching denotes the arginine-rich tract. N-terminal amino acid sequences (in single-letter code) are depicted for the wild-type and six mutants. Charged residues in the wild-type sequence are also indicated. HIV poly-A, HIV polyadenylation site. (B) CAT enzyme assay of CV1 cells after cotransfection of 0.5 µg of pDM128 with 1.0 µg of wild-type or mutant forms of pRSV-Rev. Sham, pUC118 and pCH110 only; No Rev, pDM128 and pCH110 alone. (C) Western blot detection of variant Rev proteins in transfected COS7 cells with Rev-specific antisera and radiolabeled protein A after fractionation in a denaturing 14% polyacrylamide gel. Positions of ^{14}C -labeled molecular mass standards (in kilodaltons) are indicated at right.

had *trans*-inhibitory activity, we tested the ability of the mutant plasmids to block the function of a wild-type Rev vector in our transfection assay. For comparison, two plasmids ($\Delta 78-114$ and M10) encoding C-terminal mutants were also tested. As illustrated in Fig. 5, cotransfection of increasing amounts of these C-terminal variants progressively inhibited the induction of CAT by wild-type Rev. As little as 5 µg of M10, a mutant previously shown to be strongly dominant-negative (18), was sufficient to achieve 98% inhibition of transactivation in our assay. In contrast, neither MA4 nor MA5 produced significant inhibition under any of the conditions tested. We therefore conclude that both MA4 and MA5 are recessive-negative mutations.

DISCUSSION

The Rev transactivator of HIV-1 is a small RNA-binding protein that is thought to interact with certain incompletely

FIG. 3. Defective localization of Rev proteins that contain N-terminal mutations. COS7 cells that had been transfected with wild-type or mutant pRSV-Rev were fixed in situ and then stained with a mixture of three Rev-specific primary antisera, followed by a fluorescent second antibody. Approximately 5% of cells in each population showed detectable expression of the transfected gene by this assay. The results shown are typical of two or more independent experiments with each construct.

spliced HIV-1 transcripts in the nucleus (3, 5, 34). As a result of this interaction, the transcripts are selectively exported to the cytoplasm, where they direct the synthesis of essential virion proteins (6, 8, 19, 26). The precise mechanism of Rev action remains unknown, but its importance in the viral life cycle is clear: HIV-1 proviruses that cannot express functional Rev protein also fail to generate infectious viral particles (7, 14, 27, 31, 32). It appears likely that this form of posttranscriptional control is critical for other primate retroviruses as well. Thus, similar Rev proteins are expressed by HIV-2 and by simian immunodeficiency virus (SIV) (17), and a structurally distinct protein (termed Rex) produced by human T-cell leukemia/lymphoma virus type I (HTLV-I) appears to serve a virtually identical function (12). The effect of each of these proteins is mediated by a *cis*-acting RNA target element found in transcripts from the corresponding

virus. Although their sequences are unique to each virus, some of these proteins and response elements can substitute for one another under certain conditions (17, 24, 25), implying that they may act through a common pathway.

The studies reported here are an extension of earlier work (18) which delineated two essential regions in the Rev protein of HIV-1. One of these spans the arginine-rich tract at amino acids 35 to 50 and has also been shown to extend to near residue 25; the other lies in the C-terminal half of the protein. Although mutations in either region can inactivate Rev, the resulting mutant proteins have markedly dissimilar properties. Thus, mutations in the arginine-rich region tend to be recessive negative and to disrupt localization of the protein, whereas mutations near amino acid 80 impair function without affecting localization and can exhibit a dominant-negative phenotype.

In this report, we present the results of a further mutational analysis of HIV-1 Rev. Using a reporter plasmid to detect Rev transactivation *in vivo*, we tested the effects of a series of missense mutations that together involved over 40% of the residues in the N-terminal half of the protein. While generally confirming the results of earlier studies, our data also reveal that point mutations at positions 14 to 16 or 18 to 20 can completely abolish Rev function. The N-terminal locus defined by these mutations (MA4 and MA5) coincides with a relatively basic segment of the protein in which three of seven consecutive amino acids carry positive charges. Indeed, the density of basic residues in this short segment of the protein approaches that of the nearby arginine-rich tract. Moreover, the effects of the MA4 and MA5 mutations resemble those seen with mutations in the arginine tract: the mutant proteins do not accumulate normally within nuclei and fail to act as *trans*-dominant inhibitors of wild-type Rev. In light of these similarities, we are inclined to view the N-terminal locus together with the arginine-rich tract as a single contiguous domain, spanning residues 14 to 50, that is essential for the localization and function of Rev.

When transported passively into the nucleus by fusion with the GR, Rev proteins containing certain mutations in the arginine-rich region are selectively excluded from nucleoli (Hope et al., *in press*), suggesting that the affected residues may target Rev to that organelle. Other mutations in this region, however, inactivate the Rev/GR fusion protein without affecting its localization (Hope et al., *in press*). The

FIG. 4. Function and localization of mutant Rev/GR fusion proteins in the presence of steroid hormone. (A) Structure of pRSV-Rev/GR. This vector was derived by inserting cDNA sequences encoding the steroid-binding domain of the rat GR into codon 116 of pRSV-Rev in frame, as detailed previously (Hope et al., *in press*). Hatching indicates locations of the arginine-rich tract and of the MA4 and MA5 mutations; the black rectangle marks residues 75 to 90. (B) Western blot detection of Rev and Rev/GR expression in transfected COS7 cells. Proteins were detected by using Rev-specific antisera after fractionation on a denaturing 12% polyacrylamide gel. kD, Kilodaltons. (C) Transactivation of CAT expression by mutant and wild-type Rev/GR. CV1 cells were cotransfected with 0.5 μg of pDM128, 0.25 μg of pCH110, and 1 μg of a pRSV-Rev derivative and were then grown in the continuous presence of the steroid agonist dexamethasone (10⁻⁵ M) for 36 h prior to harvest. As expected (Hope et al., *in press*), none of the Rev/GR derivatives showed detectable activity in the absence of steroid (data not shown). (D) Nucleolar localization of wild-type Rev/GR and representative mutants in the presence of dexamethasone. Proteins were detected by using primary antisera specific for the GR moiety. Sham, pUC118 and pCH110 only; No Rev, pDM128 and pCH110 alone.

FIG. 5. Lack of *trans*-dominant inhibitory activity in N-terminal mutants of the Rev protein. CV1 cells were cotransfected with 0.25 μ g of pCH110, 1 μ g of pDM128, and 5 μ g of wild-type ptk-Rev along with the indicated amounts of wild-type ptk-Rev or mutant pRSV-Rev (competitor) plasmid. CAT expression was assayed 36 h later and is expressed relative to the value observed in the absence of any competitor plasmid. Steady-state expression of Rev protein from ptk-Rev was substantially lower than that produced by an equimolar amount of pRSV-Rev (data not shown). M10 is the pRSV-Rev equivalent of a mutant derived by Malim et al. (18) and is identified as LE 78,79 DL in Fig. 1B.

latter finding provides support for the view that sequences in the arginine-rich tract form part of the RRE-binding domain of Rev, just as similar arginine-rich motifs are thought to function in a variety of eucaryotic and procaryotic RNA-binding proteins (16). In the present study, we found no evidence that sequences encoded by the first exon are required for nucleolar localization of Rev/GR. The finding that mutations MA4 and MA5 inactivate this fusion protein (Fig. 4) therefore implies that these mutations interfere directly with transactivation.

A comparison of predicted Rev protein sequences from all available HIV-1 isolates (22) reveals that, although individual residues in the vicinity of positions 14 to 20 may vary, the

FIG. 6. Conserved structure of an essential N-terminal motif in Rev proteins of primate immunodeficiency viruses HIV-1, HIV-2, and SIV. Predicted sequences are shown for amino acids 12 to 23 of HIV-1 Rev and for corresponding residues in HIV-2 and SIV Rev proteins. For each viral species, a sequence from one representative strain is depicted at the top, and any alternative amino acids found in other strains are listed below (in arbitrary order) for each position. Positively charged residues are shown by open letters, and the proposed conservation of these residues is indicated by dashed-line boxes. Sequences and alignments are based on data compiled in reference 22 and comprise 13 North American and Zairean isolates of HIV-1, seven isolates of HIV-2, and three isolates of macaque or sooty mangabey SIV. The functional properties of many of these isolates have not been determined.

overall structure is conserved (Fig. 6). Three positively charged residues, separated from one another by pairs of nonpolar amino acids, are predicted at corresponding positions in every known HIV-1 sequence. Interestingly, Rev sequences from HIV-2 and closely related strains of SIV also predict a very similar organization in this region but are distinguished by the presence of additional charged residues at several positions. By contrast, we can identify no recognizable counterpart of this N-terminal motif in the HTLV-I Rex protein. One obvious possibility is that, when present, the basic side chains in this region could contribute to RNA binding through strong electrostatic interactions. This might account for the observation (17) that a fusion protein formed by linking the first exon of HIV-1 *rev* with the second exon of HIV-2 *rev* can transactivate through the HIV-1 RRE, whereas the HIV-2 Rev protein itself cannot. It is also consistent with reports that hybrid HIV-1 proteins containing only the second *rev* exon are partially (2) or completely (2, 28) defective in transactivation. We therefore propose that this novel N-terminal motif may be a principal determinant of RRE-binding specificity in Rev proteins from the primate immunodeficiency viruses.

The recent development of *in vitro* RRE-binding assays (3, 5, 13, 20, 34) will permit a direct test of this hypothesis. A more speculative possibility is that this conserved motif mediates specific protein-protein interactions that may be essential for transactivation *in vivo*. The existence of such interactions, however, has not yet been demonstrated.

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