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Steroid–receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: Mapping cryptic functions of the arginine-rich motif

(acquired immunodeficiency syndrome/glucocorticoid receptor)

THOMAS J. HOPE, XIAOJIAN HUANG, DAVID MCDONALD, AND TRISTRAM G. PARSLOW*

Departments of Pathology and of Microbiology and Immunology, University of California, San Francisco, CA 94143

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ABSTRACT **The human immunodeficiency virus type 1 (HIV-1) transactivator Rev is a nuclear protein that regulates expression of certain HIV-1 transcripts by binding to an RNA target element (the RRE) present in these transcripts. A short arginine-rich sequence in Rev contains the signals required to direct this protein into nuclei, where it associates preferentially with nucleoli. We created a steroid-inducible transactivator by fusing Rev with the steroid-binding domain of the glucocorticoid receptor (GR). This Rev/GR protein remains inactive in the cytoplasm when steroids are absent, but it enters the nucleus and initiates transactivation within minutes after exposure to dexamethasone. Although the GR moiety is sufficient to transport Rev/GR into nuclei, mutation of certain residues in the arginine-rich region blocks nucleolar localization and also inhibits transactivation. We find that other mutations in this region, however, can abolish the function of Rev/GR without affecting its localization; the latter phenotype may reflect a specific defect in binding of the RRE.**

The *rev* gene of human immunodeficiency virus type 1 (HIV-1) encodes a 116-amino acid regulatory protein that transactivates the expression of other essential HIV-1 genes (1, 2). Localized in the nuclei of infected cells, where it associates preferentially with nucleoli (3), Rev acts at the posttranscriptional level by inducing the export of incompletely spliced viral mRNAs to the cytoplasm (1, 2, 4–8). The mechanism of this transactivation is not fully understood, but it is thought to depend on the binding of Rev protein to an array of RNA hairpin loops (termed the Rev response element, RRE) situated within the target HIV-1 transcripts (9–14).

The signals that direct Rev protein into nuclei are contained in a short basic region of the protein (approximately amino acids 25–50) that is rich in arginine residues (15, 16). Mutations in this basic region inhibit the nuclear accumulation of Rev and produce a concomitant defect in transactivation, implying that Rev must enter the nucleus to exert its effects. Although recent reports suggest that this region also harbors sequences that target Rev to nucleoli (17, 18) or participate in binding of the RRE (19), it has been difficult to evaluate these activities by conventional mutagenic analysis *in vivo* because of the need to maintain efficient nuclear translocation. We now describe studies of a Rev fusion protein whose entry into the nucleus is controlled by sequences from the glucocorticoid receptor (GR). This Rev/GR protein exhibits steroid-dependent Rev activity and thus provides the basis for an inducible system in which to study the dynamics of Rev transactivation. Moreover, because the GR moiety is sufficient to direct this protein into nuclei, mutational analysis of Rev/GR has enabled us to evaluate the functions of the basic region independently of any effect on nuclear translocation. Our findings illuminate the critical and complex role of this region in the localization and function of Rev.

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MATERIALS AND METHODS

Plasmid Constructions. The reporter plasmid pDM128 was derived from pSV9B, which transcribes the genome of HIV-1 strain SF2 under control of the simian virus 40 (SV40) promoter and enhancer (20). We replaced HIV-1 nucleotides (nt) 640–5863 with an *Xba* I linker; we then used oligonucleotide-directed mutagenesis (21) to introduce an *Eco*RI site at nt 9722, to delete nt 8400–8915, and to replace the *rev* and *env* start codons by unique *Bcl* I and *Not* I sites at nt 5979 and 6233, respectively. The *Not* I/*Dra* III fragment was replaced by the 780-base-pair chloramphenicol acetyltransferase (CAT) coding sequence, and the entire transcription unit (*Sal* I/*Eco*RI) was then subcloned into pUC18 to produce pDM128 (see Fig. 1). pRSV-Rev contains the Rev coding sequence from HIV strain BRU (22), the Rous sarcoma virus (RSV) promoter, and HIV-1 polyadenylylation signals (nt 9575–9694) in pUC118; mutations were introduced into pRSV-Rev by oligonucleotide-directed mutagenesis and were confirmed by DNA sequence analysis. pRSV-Rev/GR was prepared by converting codon 116 of pRSV-Rev to a *Bgl* II site (Asp replaces Glu) and then ligating codons 511–795 of the rat GR gene (23) into this site in-frame.

Transfection and Expression Assays. Calcium phosphate transfections, isolation of cytoplasmic RNA, and Northern and Western blot analyses were essentially as described (24). Each transfection for CAT assay included the indicated Rev and reporter plasmids, along with 0.25 μg of the SV40/β-galactosidase expression plasmid pCH110 (25) and sufficient pUC18 for 20 μg of total DNA per 10-cm plate; CAT assays were normalized to the expression of β-galactosidase activity from pCH110 as described (25), providing an internal control for transfection efficiency and for nonspecific effects on the SV40 promoter.

Immunofluorescence Analysis. *In situ* immunocytochemistry was performed as described (26), using cells grown on coverslips, 36 hr after transfection with 10 μg of pRSV-Rev (or its derivatives) per 3.5-cm plate. The primary rabbit anti-GR antiserum (a gift of R. Sweet, Smith Kline & French) had been raised against the steroid-binding domain of the mouse GR, and cross-reacts with the rat GR. The Rev antiserum (a gift from B. R. Cullen, Duke University) was a

Abbreviations: CAT, chloramphenicol acetyltransferase; Dex, dexamethasone; GR, glucocorticoid receptor; HIV-1, human immunodeficiency virus type 1; nt, nucleotide(s); RRE, Rev response element; RSV, Rous sarcoma virus; SV40, simian virus 40.

*To whom reprint requests should be addressed at: Department of Pathology, Box 0506, University of California, San Francisco, CA 94143-0506.

combination of two rabbit anti-peptide antisera specific for amino acids 1–20 and 27–51 of Rev (3), respectively, each used at 1:800 dilution. The secondary antiserum was rhodamine-conjugated goat anti-rabbit IgG.

RESULTS

We assayed Rev function in a transient transfection system by using a reporter plasmid (pDM128) derived from the *env* region of HIV-1 (Fig. 1*A*). The transcripts produced by pDM128 harbor a single intron containing the CAT coding sequence, which is excised when the RNA is spliced. Cells transfected with pDM128 alone express only the spliced transcripts in the cytoplasm (Fig. 1*B*) and thus yield only trace levels of CAT enzyme activity (Fig. 1*C*). Cotransfection with a functional Rev expression vector (pRSV-Rev), however, permits the unspliced transcripts to enter the cytoplasm, increasing CAT activity 75- to 100-fold (Fig. 1 *B* and *C*). This response can be abolished by various mutations in the coding sequence of pRSV-Rev (Fig. 1*C*, lanes 12 and 13), implying the need for a functional Rev protein.

Although the steroid-binding domain of the GR contains nuclear translocation signals, it enters the nucleus only when steroid is present, perhaps because the unliganded domain tends to associate with a specific protein in the cytoplasm (26, 27). This domain of the GR lacks the DNA-binding and transcriptional regulatory activities of the intact steroid receptor but is sufficient to confer dominant hormonal control onto the localization of fusion proteins (26–29). We therefore constructed an expression vector (pRSV-Rev/GR) that encodes a fusion protein in which the steroid-binding domain of the rat GR is appended to the C terminus of Rev (Fig. 2*A*). As shown in Fig. 2*B*, transactivation by this chimeric Rev/GR protein proved to be strictly hormone dependent. When grown in the continuous presence of the steroid agonist dexamethasone (Dex), cells transfected with as little as 0.5 μg of pRSV-Rev/GR expressed CAT activity at levels nearly equal to those achieved with pRSV-Rev; in contrast, even larger amounts of pRSV-Rev/GR had no effect when the hormone was absent. By adding Dex at various times before harvesting the cells, we found that CAT expression was detectably increased within 30 min after addition of the hormone and thereafter increased linearly to achieve steady-state levels 10–12 hr later (Fig. 2*C*).

In situ immunofluorescence staining, using antisera specific for either the GR or Rev moieties, confirmed that the hormonal activation of Rev/GR coincided with a dramatic shift in localization of the protein (Fig. 2*D*). In the absence of Dex, immunoreactive Rev/GR was abundantly expressed in the cytoplasm but was largely excluded from nuclei; within 20 min after exposure to Dex, however, the fusion protein became almost entirely intranuclear. Rev/GR was distributed throughout the nucleoplasm in Dex-treated cells but was particularly abundant in nucleoli, mimicking precisely the distribution of wild-type Rev (3). Thus, presynthesized Rev/GR is retained in the cytoplasm when steroids are absent but is rapidly transported to the nucleus and nucleoli and initiates transactivation within minutes after exposure to Dex.

To determine whether nuclear translocation mediated by the GR moiety could compensate for mutations in the Rev basic domain, we used oligonucleotide-directed mutagenesis to derive a series of nine variants of pRSV-Rev/GR that encoded mutant forms of the fusion protein (Fig. 3*A*). One of these mutants (MBΔ) harbored a deletion of amino acids 38–50; the others were missense mutants (designated MB1–MB8) that together affected half the residues in the basic

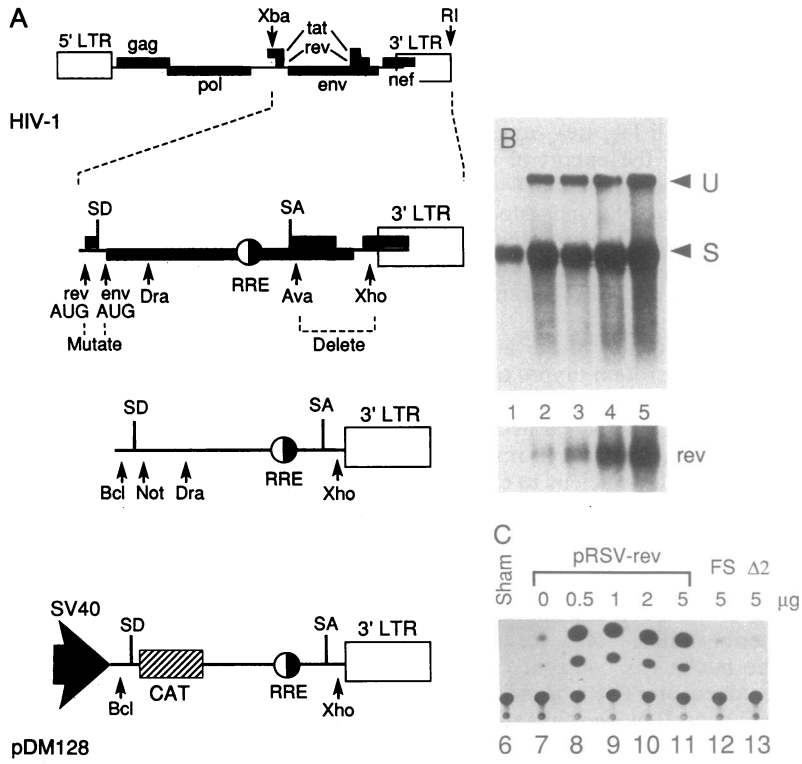

FIG. 1. A transient transfection assay for Rev activity. (*A*) Derivation of Rev-dependent reporter plasmid pDM128 from the HIV-1 genome. The RRE (9) and the splice donor (SD) and acceptor (SA) sites are indicated. (*B*) Cytoplasmic RNA expression in COS7 cells transfected with 0.5 μg of pDM128 either alone (lane 1) or along with 2, 5, 10, or 20 μg of pRSV-Rev (lanes 2–5). Duplicate 5-μg aliquots of RNA were probed for spliced (S) and unspliced (U) pDM128 transcripts by using a 3′ long terminal repeat (LTR) probe (*Upper*) or for *rev* coding sequences (*Lower*). The 3′ LTR probe was HIV-1 nt 8915–9575; the Rev probe was a full-length cDNA. (*C*) CAT enzyme assay of CV1 cells cotransfected with 1 μg of pDM128 and the indicated amounts of wild-type (lanes 7–11) or mutant forms of pRSV-Rev (lanes 12 and 13). FS, frameshift at codon 58 as described (4); Δ2, in-frame deletion of codons 38–50.

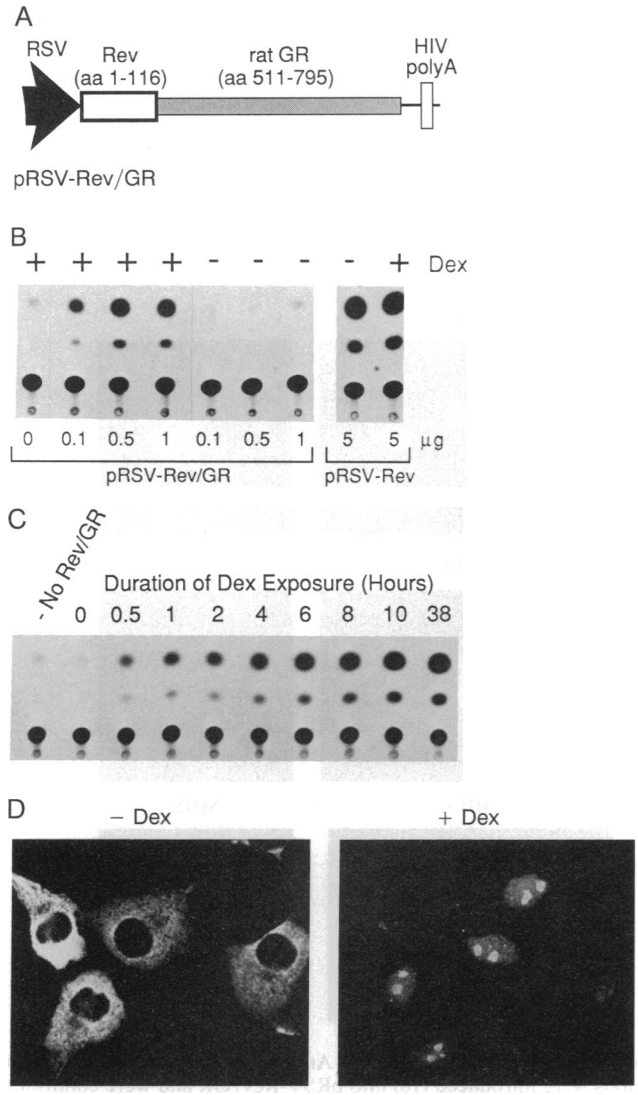

FIG. 2. Hormonal control of the function and localization of a Rev/GR fusion protein. (*A*) Structure of the fusion gene in pRSV-Rev/GR. aa, Amino acids. (*B*) Steroid-dependent transactivation by Rev/GR. CV1 cells were cotransfected with 1 μg of pDM128 and the indicated amounts of pRSV-Rev/GR or pRSV-Rev and then grown in the presence or absence of Dex for 40 hr prior to CAT assay. (*C*) Time course of CAT induction after hormonal activation of Rev/GR. CV1 cells were cotransfected with 0.5 μg of pDM128 and 1 μg of pRSV-Rev/GR and were harvested 40 hr later for CAT assay. Dex had been added to some plates at the indicated times before harvest. No Rev/GR, pDM128 alone (Dex added 38 hr before harvest). (*D*) *In situ* immunofluorescence localization of Rev/GR by using anti-GR antibodies. CV1 cells were either grown in the absence of Dex (−Dex) or received 10 μM Dex for 1 hr before harvest (+Dex). Identical results were obtained by using antisera specific for Rev. CV1 cells contain no detectable endogenous GR (26).

domain. CV1 cells were transfected with each of the plasmids, and protein extracts from these cells were analyzed by Western blotting using antisera specific for Rev. As illustrated in Fig. 3*B*, each of the nine mutant plasmids gave rise to a single immunoreactive protein of the expected molecular mass, and each of these proteins was expressed at a steady-state level comparable with that of wild-type Rev/GR. Nevertheless, when assayed for the ability to induce CAT expression from pDM128, six of the mutants proved to be completely defective in transactivation, and the remaining three exhibited only partial activity (Fig. 3*C*). The function of Rev/GR thus appeared to remain highly dependent on sequences in the basic domain.

Table 1. Summary of phenotypes of wild-type and mutant Rev/GR proteins

Rev/GR fusion protein	Nuclear translocation	Nucleolar localization	Transactivation
Wild-type	+	+	+++
MBΔ	+	−	−
MB1	+	+	−
MB2	+	±	−
MB3	+	−	−
MB4	+	±	+
MB5	+	±	+
MB6	+	+	+
MB7	+	+	−
MB8	+	+	−

All data are based on transfected CV1 cells grown in the presence of Dex. Transactivation was assayed by using pDM128: +++, 90–100%; +, 5–50%; −, 0–5% CAT induction as compared to wild-type REV/GR in three independent experiments. Because suprathreshold amounts of the Rev plasmid were used, this scale overestimates Rev activity. Nucleolar localization was evaluated by immunofluorescence; three independent studies of each construct were scored by a viewer who was unaware of the identity of any mutant. + or − indicates that nucleoli were positive or negative, respectively, in essentially all transfected cells; ± denotes a roughly equal admixture of cells with positive and negative nucleoli in the transfected population.

Immunofluorescent staining *in situ* revealed that each of the Rev/GR mutants was efficiently transported into the nucleus after exposure to Dex. As illustrated in Fig. 3*D*, however, several of these mutants exhibited an anomalous intranuclear distribution in which the fusion protein was selectively excluded from nucleoli. This localization defect was most apparent for mutants MBΔ and MB3, but it occurred to a lesser degree in mutants MB2, MB4, and MB5 (Table 1). Sequences required for the nucleolar accumulation of Rev thus appear to lie in the vicinity of arginine residues 41–43, a region previously reported to be both necessary and sufficient for nuclear translocation (15, 16).

Other mutant fusion proteins, however, showed a pattern of localization indistinguishable from that of wild-type Rev/GR, with intensely fluorescent nucleoli appearing against a lower level of diffuse nucleoplasmic staining (Fig. 3*D*). This pattern was observed with the partially functional mutant MB6, as well as with three mutants (MB1, MB7, and MB8) that were completely defective in transactivation (Table 1). Mutations that involve residues 27–29 or 46–50 in the basic region thus can completely abolish the *in vivo* function of Rev/GR without appreciably affecting its stability or nucleolar localization.

DISCUSSION

We have described a chimeric Rev/GR protein whose localization and function are controlled by steroid hormones. Analogous fusion proteins have been used by other investigators to confer hormonal control onto transcription (28) or oncogenesis (29); our studies demonstrate that this same approach can be applied to posttranscriptional regulatory factors. The conditional function of Rev/GR permits rapid and synchronous induction of Rev activity and thus provides a unique opportunity to examine the sequence of events that occur during transactivation. Our initial studies indicate, for example, that Rev/GR yields detectable transactivation within minutes after exposure to Dex (Fig. 2*C*), implying that Rev affects mRNA expression through a relatively direct mechanism.

Mutational analysis of this fusion protein has also enabled us to dissect the functions of a distinctive arginine-rich region spanning amino acids 25–50 of Rev. This region is known to

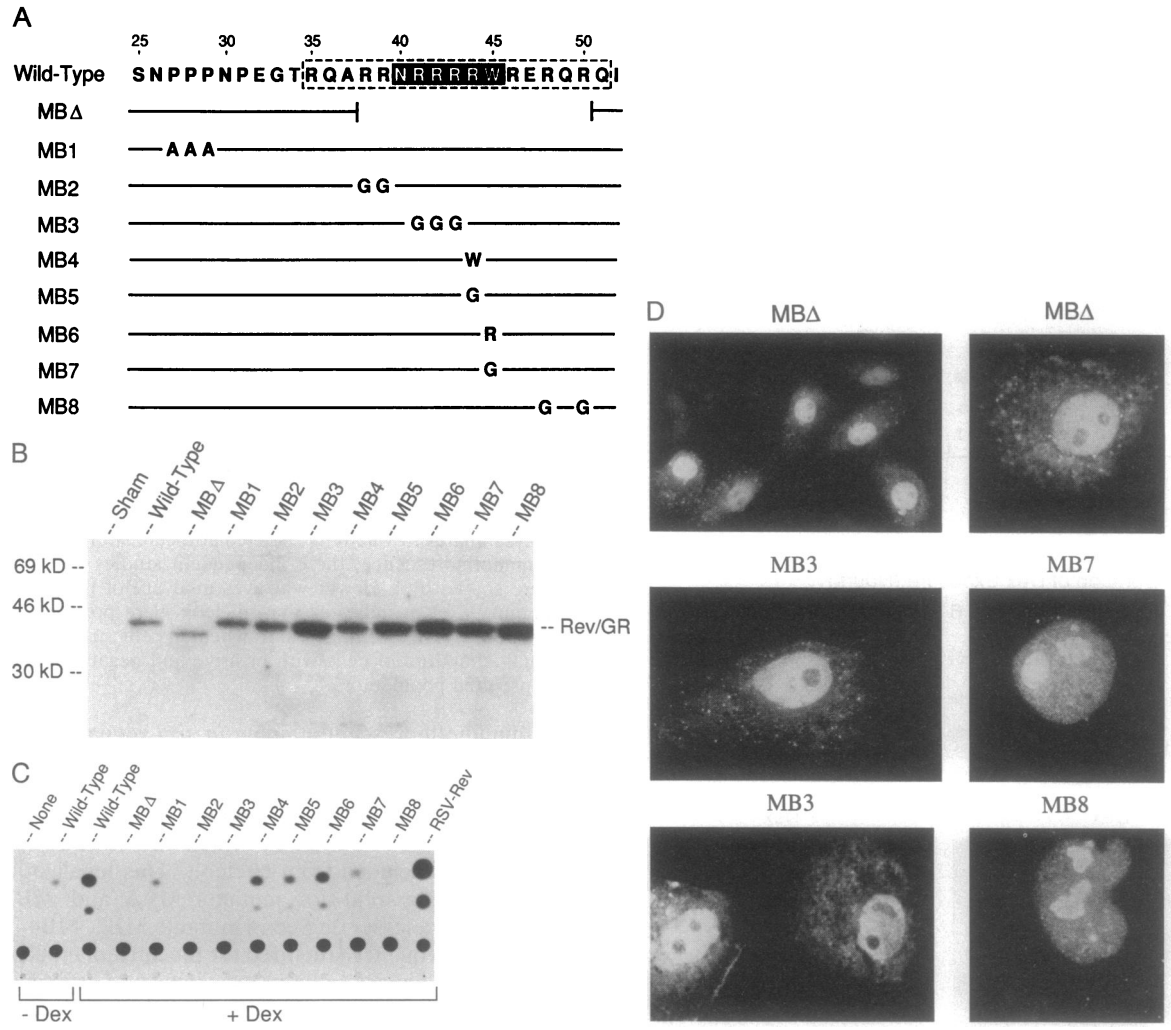

FIG. 3. Mutations in the basic domain of Rev/GR affect nucleolar association and transactivation. (*A*) Amino acid sequence (single-letter code) of the basic domain in wild-type Rev/GR and in nine mutants. Mutations were introduced (18) into pRSV-Rev/GR and were confirmed by DNA sequencing. Boundaries of the domain are approximate (15) and encompass a hexapeptide (white lettering) that is sufficient to direct nuclear translocation of a heterologous protein (16) and an arginine-rich tract (dotted lines). (*B*) Western blot detection of mutant Rev/GR proteins in transfected COS7 cells by using Rev-specific antisera and ^{125}I-labeled protein A after fractionation on a denaturing polyacrylamide gel. (*C*) Transactivation function of mutant Rev/GR proteins assayed by cotransfection with 1 μg of pDM128 in the continuous presence of Dex. Mutant MB5 gave no detectable transactivation in either of two similar studies. (*D*) Immunofluorescence localization of representative Rev/GR mutants in transfected CV1 cells 2 hr after exposure to Dex by using primary antiserum specific for the GR moiety.

contain signals for nuclear translocation (15, 16), and these signals presumably help to maximize the effect of the relatively small amount of Rev present in virally infected cells. Because of its small size (13.5 kDa), however, the Rev protein might be expected to enter the nucleus by passive diffusion alone, as nuclear pores freely admit molecules of up to 70 kDa (30). Consistent with this view, earlier studies have shown that Rev variants containing mutations in the arginine-rich region are not completely excluded from the nucleus, but rather tend toward equal concentrations in the nucleus and cytoplasm (15). Nevertheless, such mutants fail to induce transactivation even when vastly overexpressed, implying that the importance of this region extends beyond its role in nuclear translocation.

In the present study, we demonstrate that certain residues within the arginine-rich region are necessary for the association of Rev/GR with nucleoli. While confirming earlier reports of a nucleolar localization signal in this region of Rev (17, 18), analysis of Rev/GR has permitted us to map the residues involved more precisely and without any confounding effects on nuclear translocation. Our results indicate that the essential sequences are confined to a discrete cluster of residues at or near positions 41–43—a distinctly different location than has previously been proposed (18). These residues coincide with a hexapeptide element in Rev (amino acids 40–45) that reportedly is sufficient to target a heterologous protein to the nucleus (16), suggesting that this element may serve a bifunctional role in both nuclear and nucleolar localization. A similar overlap of targeting signals has been observed in the human T-cell leukemia virus type 1 protein Rex, whose association with nucleoli depends in part on a signal formed by two adjacent nuclear translocation motifs (31).

Because nucleoli are not membrane bounded, the association of Rev with this organelle presumably results from binding of the protein to some component of the nucleolus, rather than from a translocation process *per se*. In our mutational analysis, this binding appears to correlate with function: mutant Rev/GR proteins that do not associate with nucleoli (MBΔ and MB3) are profoundly defective in transactivation, even when expressed abundantly throughout the remainder of the nucleoplasm. Significantly, however, our study also reveals that mutations at several other sites in the basic region can abolish transactivation independently of any effect on protein localization (Table 1). This mutant phenotype strongly implies that, in addition to its roles in nuclear

translocation and nucleolar association, the basic region contributes to a third distinct function that is essential for Rev activity. Our data do not specify the nature of this third function but are consistent with the hypothesis (15) that sequences in the arginine-rich region form part of the RRE-binding site of Rev. Although as yet unproven, this hypothesis is supported by the recent demonstration (19) that arginine-rich motifs in certain phage proteins can mediate binding to RNA stem–loop structures comparable to those in the RRE. Moreover, similar arginine motifs are also present in a variety of prokaryotic and eukaryotic ribosomal proteins, where they presumably serve to bind secondary structures in ribosomal RNA (19). These observations suggest that the arginine-rich sequence of Rev might have an affinity both for the RRE and for the abundant ribosomal RNA in nucleoli, although the physiological importance of the latter interaction is unknown. Our data suggest that certain mutations in the basic region (MBΔ and MB3) can inhibit binding of both these ligands. In contrast, the unusual phenotype of Rev/GR mutants MB1, MB7, and MB8 is likely to reflect a specific defect in binding of the RRE and provides evidence that this binding is essential for transactivation *in vivo*.

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