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Mutational Analysis of the Human T-Cell Leukemia Virus Type I *trans*-Acting *rex* Gene Product

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Expression of the human T-cell leukemia virus type I (HTLV-I) *rex* gene is a prerequisite for the expression of the retroviral structural proteins. We have generated internal deletion mutants of this 27-kDa nucleolar *trans*-acting gene product to define functional domains in the Rex protein. The phenotype of the various mutant proteins was tested on the homologous HTLV-I *rex* response element sequence and the heterologous human immunodeficiency virus type 1 (HIV-1) *rev* response element sequence. Our results indicate that a region between amino acid residues 55 and 132 in the 189-amino-acid Rex protein is required for Rex-mediated *trans* activation on both retroviral response element sequences. In addition, substitution of the Rex nuclear localization signal by a sequence of the HIV-1 *rev* gene product targets the Rex protein to the correct subcellular compartment required for Rex function.

Replication of human T-cell leukemia virus type I (HTLV-I), the causative agent of adult T-cell leukemia (16, 30, 31, 38), is dependent on the function of the virus-encoded *trans*-acting regulatory protein Rex. The Rex protein recognizes the viral mRNAs via a *cis*-acting sequence, named the *rex* response element (RxRE), located in the 3′ long terminal repeat (LTR) of HTLV-I (15, 17, 18, 34). Rex function results in the cytoplasmic accumulation of unspliced (*gag/pol*) or singly spliced (*env*) viral mRNAs which serve either as the viral genome or as the template for translation into the structural proteins (9, 13, 15, 17, 18, 27, 32, 36). The *trans*-acting protein Rev serves similar functions in the related lentivirus human immunodeficiency virus type 1 (HIV-1) (8, 10, 12, 25). Rev interacts directly with its target sequence, the *rev* response element (RRE), present in HIV-1 *env* gene mRNA (6, 7, 11, 14, 25, 26, 28, 39). The exact mechanism of HTLV-I Rex and HIV-1 Rev function is still unclear.

The activity of HTLV-I Rex and HIV-1 Rev is dependent on the correct nuclear or nucleolar localization of both proteins (3, 5, 10, 20, 27, 29). In each case, a short stretch of basic amino acids was identified which serves as a nuclear localization signal responsible for the targeting of these *trans* activator proteins to the nucleolus (3, 21, 23, 27, 35). In addition, these nuclear localization signals are able to impose nucleolar accumulation on heterologous proteins (3, 21, 35, 37).

Despite the fact that there is no obvious sequence homology between Rex and Rev, it has been shown that the HTLV-I *rex* gene product is able to rescue the replication of a defective HIV-1 provirus mutated in the *rev* gene (33). While Rex is able to exert its phenotype on both the homologous HTLV-I RxRE sequence and the heterologous HIV-1 RRE sequence, the HIV-1 Rev protein functions only on the homologous HIV-1 element (1, 9, 13, 19).

In the present study we first generated serial internal HTLV-I Rex deletion mutants in order to locate regions in this protein required for Rex function. For this, we exploited a series of Rex missense substitution mutants (RexM1 to RexM28, Fig. 1) (2) which introduced a *Bgl*II restriction enzyme recognition site in the same translational frame in the *rex* gene sequence. For example, the mutant Rex1Δ4 was constructed by fusing the appropriate DNA fragments of RexM1 and RexM4, thereby deleting amino acid residues 22 to 44 (Fig. 1) of the Rex amino acid sequence. In order to ensure a correct nucleolar accumulation of the HTLV-I Rex protein (20, 27, 35), our initial deletions did not affect amino acid residues 1 to 21, which serve as the nuclear localization signal of the Rex *trans*-acting protein (27, 35). All mutant *rex* genes used in this study are summarized in Table 1.

All genes used were transfected into COS cell cultures and expressed as described previously (4, 24), with the cytomegalovirus immediate-early promoter (provided by B. Fleckenstein). The relative level of expression of mutants with deletions close to the Rex amino terminus, as assayed by immunoprecipitation of ^{35}S-cysteine-labeled cultures (2), is presented in Fig. 2A. The polyclonal antiserum Rex173/189 specifically recognizes the various Rex proteins (Fig. 2A, lanes 3 to 13). No Rex-specific signal was detected in a negative control experiment (Fig. 2A, lane 1). Clearly, the wild-type Rex protein is migrating, as expected, at a relative molecular mass of 27 kDa (Fig. 2A, lane 3) (20). In contrast, the increasing electrophoretic mobility of the various mutant proteins reflects the increasing internal deletions in the corresponding *rex* genes (Fig. 2A, lanes 4 to 13).

To test the *trans* activation capacity of the various Rex mutant proteins on the homologous HTLV-I RxRE and on the heterologous HIV-1 RRE, we performed cotransfection experiments, followed by Tat-specific immunoprecipitation analysis (2) with a murine monoclonal antibody raised against recombinant Tat protein from *Escherichia coli*. This antibody specifically recognizes the one- and two-exon forms of the Tat protein (Fig. 2B and C) expressed from the RRE-specific indicator construct gTat (24). The 86-amino-acid form of the HIV-1 Tat protein is translated from fully spliced *tat* mRNA. Coexpression of functional Rev or Rex protein results in the formation of the 72-amino-acid form of the Tat protein due to the cytoplasmic expression of unspliced *tat* mRNA (1, 13, 24, 33). Substitution of the HIV-1

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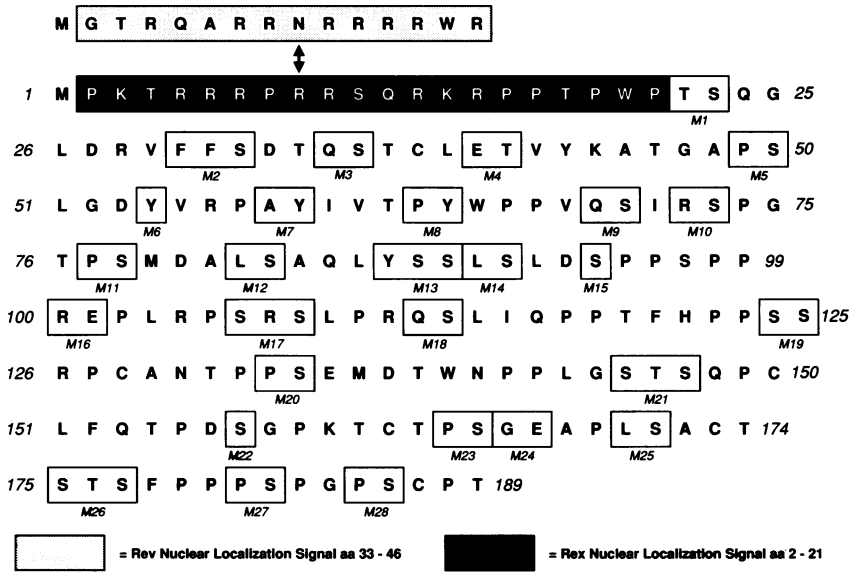

FIG. 1. Amino acid sequence of the HTLV-I Rex *trans*-activator protein. A set of missense substitution mutants (indicated by boxes [2]) were used to generate the serial internal deletion mutants. In Rex[vNLS], the wild-type nuclear localization signal (solid box) was substituted by a sequence of the HIV-1 Rev protein covering the Rev nuclear localization signal (shaded box).

RRE sequence in the gTat plasmid by the HTLV-I RxRE sequence results in the construct gTat-RxRE and provides a Rex-specific assay system (2). The phenotypes of the various Rex mutant proteins are shown in Fig. 2B and C.

Clearly, the wild-type Rex *trans*-activator protein is active on both the RxRE sequence and the RRE sequence, as indicated by the expression of the 72-amino-acid form of the Tat protein (Fig. 2B and C, lane 3 versus lane 1). In contrast, the HIV-1 Rev protein acts only on its homologous RRE sequence (Fig. 2B and C, lane 2 versus lane 1). Inspection of Fig. 2B reveals that the Rex mutants Rex1Δ2 to Rex1Δ6 were functional on the RxRE sequence (Fig. 2B, lanes 4 to 8), while mutant Rex1Δ7 displayed reduced Rex activity (Fig. 2B, lane 9). Extension of the internal deletion caused a total loss of *trans*-activation capacity, starting with mutant Rex1Δ8 to mutant Rex 1Δ11 (Fig. 2B, lanes 10 to 13). Although the wild-type Rex protein is less active than the wild-type Rev protein on the RRE sequence (1, 2, 13, 33), the Rex mutants encoded by Rex1Δ3 to Rex1Δ6 clearly displayed activity on the HIV-1 element (Fig. 2C, lanes 5 to 8). No activity could be detected for the Rex proteins encoded by Rex1Δ2 and Rex1Δ7 to Rex1Δ11 (Fig. 2C, lanes 4, 9 to 13).

The effect of internal deletion mutations in the more carboxy-terminal part of the Rex protein was tested by Rex-specific immunoprecipitation with the polyclonal rabbit antiserum Rex99/119, which was raised against a peptide containing amino acids 99 to 119 of the Rex protein (not shown). The function of these mutants on the RxRE and the RRE is summarized in Table 1 and revealed no significant differences in the assay system used. The Rex mutants encoded by Rex26Δ28 to Rex20Δ28 displayed activity on both target sequences (Table 1). No activity was detectable for the mutants Rex19Δ28 to Rex16Δ28 (Table 1). Therefore, a region located between the mutations RexM6 and RexM20 (Fig. 1) is important for Rex function in vivo.

Both the HTLV-I Rex and the HIV-1 Rev proteins are nuclear proteins which are concentrated in the nucleoli (22, 27, 35). The accumulation of Rex and Rev in the nucleolar compartment is required for the *trans*-acting phenotype (3, 27, 37). We next compared the subcellular localization of the HIV-1 Rev and the HTLV-I Rex proteins in order to investigate the correlation of this localization with biological activity. For this, we engineered a mutant version of the *rex* gene named Rex[vNLS]. In this *rev-rex* hybrid gene, the Rex wild-type nuclear localization signal (amino acid residues 1 to 19 [35]) was replaced by a sequence encompassing the Rev nuclear localization signal (Rev amino acid residues 33 to 46, Fig. 1) (3, 29, 37). Transfection of COS7 cell cultures and indirect immunofluorescence studies were performed as described previously (2). These experiments revealed a nucleolar concentration of the protein encoded by Rex[vNLS] indistinguishable from the signal obtained for the wild-type Rev and wild-type Rex proteins (Fig. 3C versus Fig. 3A and B).

Amino-terminal internal deletion mutants of the gene encoded by Rex[vNLS] (Table 1) were generated to investigate the effect of the heterologous Rev nuclear localization signal with respect to Rex-mediated *trans* activation. The Rex-specific antiserum Rex173/189 was able to specifically immunoprecipitate the various hybrid proteins (data not shown). Biological activity on the homologous HTLV-I RxRE sequence and on the heterologous HIV-1 RRE sequence was detectable, as summarized in Table 1. The internal deletion mutants encoded by Rex[vNLS]1Δ3 to Rex[vNLS]1Δ6 displayed somewhat reduced activity on the RxRE sequence (Table 1), while an intermediate activity was detected for mutant Rex1Δ2 (Table 1). As observed previously for the wild-type Rex protein on the RRE sequence, the mutants Rex[vNLS]1Δ3 to Rex[vNLS]1Δ6 were functional, while mutant Rex[vNLS]1Δ2 was inactive (Table 1).

Clearly, our results with mutants Rex1Δ6 and Rex[vNLS]1Δ6 indicate that, independent of the nuclear localization signal used, sequences carboxy-terminal of this region (amino acid position 55; Fig. 1) are important for Rex-mediated *trans* activation on both target elements.

In this report, we generated internal deletion mutants of the *rex* gene product and tested them for their *trans*-activa-

TABLE 1. *rex* mutant clones and *trans*-activation capacity[a]

Designation	Amino acids deleted	*trans*-Activation activity	
		On RxRE	On RRE
Rex (wild type)		++	++
Rev (wild type)		−	++
Rex1Δ2	22–32	++	−
Rex1Δ3	22–36	++	++
Rex1Δ4	22–41	++	++
Rex1Δ5	22–50	++	++
Rex1Δ6	22–54	++	++
Rex1Δ7	22–59	+	−
Rex1Δ8	22–64	−	−
Rex1Δ9	22–70	−	−
Rex1Δ10	22–73	−	−
Rex1Δ11	22–78	−	−
Rex26Δ28	175–186	++	++
Rex24Δ28	166–186	++	++
Rex22Δ28	157–186	++	++
Rex20Δ28	133–186	+	+
Rex19Δ28	124–186	−	−
Rex18Δ28	112–186	−	−
Rex16Δ28	100–186	−	−
Rex[vNLS]		+	++
Rex[vNLS]1Δ2	22–32	±	−
Rex[vNLS]1Δ3	22–36	+	++
Rex[vNLS]1Δ4	22–41	+	++
Rex[vNLS]1Δ5	22–50	+	++
Rex[vNLS]1Δ6	22–54	+	++
Rex[vNLS]1Δ7	22–59	−	−
Rex[vNLS]1Δ8	22–64	−	−
Rex[vNLS]1Δ9	22–70	−	−

[a] Relative *trans* activation on the RxRE and RRE sequences compared with wild-type activity. Symbols: ++, full activity; +, reduced activity; ±, intermediate activity; −, no activity. In most mutants, the amino acid residues aspartic acid and leucine (Asp-Leu) are inserted at the location of the internal deletion. However, in mutant Rex26Δ28, the amino acid residues leucine-aspartic acid-leucine (Leu-Asp-Leu) are inserted, and in mutant Rex22Δ28, the amino acid residue leucine (Leu) is inserted. The Rex nuclear localization signal, located in the amino-terminal region of the Rex protein (amino acid residues 2 to 21), is substituted by a region of the HIV-1 Rev protein (amino acid residues 33 to 46) in the Rev-Rex hybrid mutant Rex[vNLS].

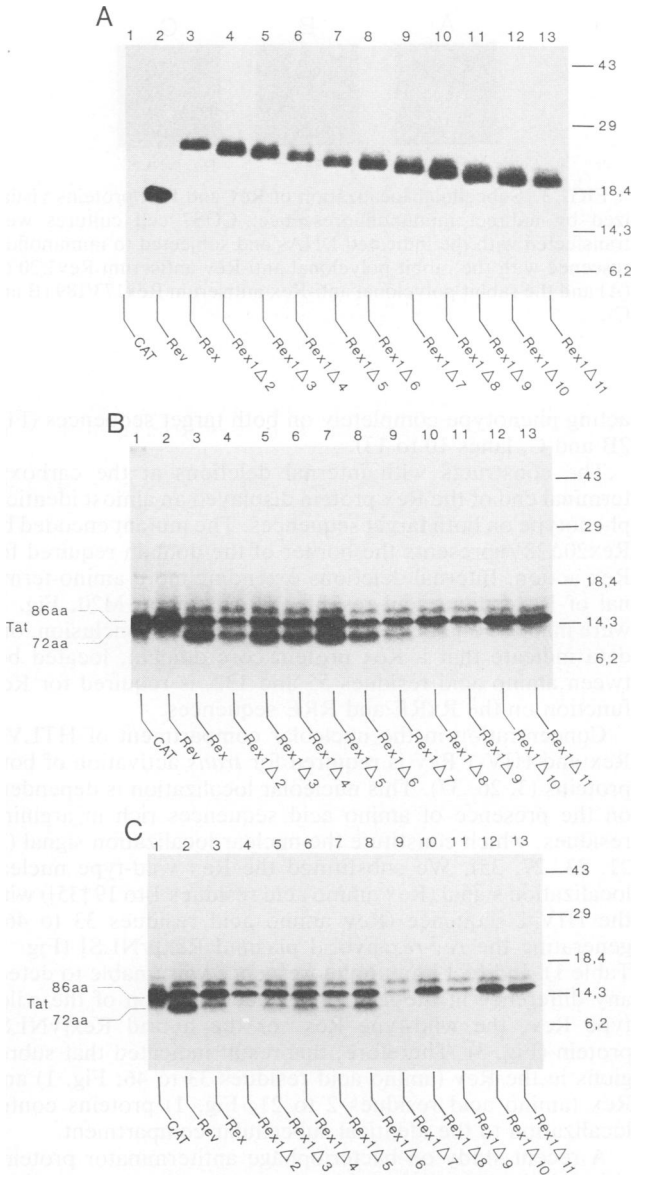

FIG. 2. Characterization of amino-terminal Rex internal deletion mutants. (A) Analysis of protein expression by immunoprecipitation analysis. COS cells (2.5×10^5 per 35-mm dish) were transfected (4) with 0.25 μg of the indicated DNAs. At 60 h posttransfection, cells were radiolabeled with [^{35}S]cysteine, followed by immunoprecipitation with an anti-Rex (Rex173/189; lanes 1 and 3 to 13) or anti-Rev (5) (lane 2) antiserum. Precipitated proteins were resolved on discontinuous SDS–13% polyacrylamide gels and visualized by autoradiography. Molecular mass standards (in kilodaltons) are shown on the right. The wild-type Rex protein migrates at 27 kDa (lane 3), and the wild-type Rev protein migrates at 19 kDa (lane 2). A vector expressing the *cat* gene served as a negative control, indicating the specificity of the anti-Rex antiserum used. (B) Function of the various Rex mutant proteins on the homologous HTLV-I RxRE sequence. COS cells were cotransfected with 0.2 μg of the Tat indicator plasmid gTat-RxRE and 0.1 μg of the indicated DNAs. Tat-specific immunoprecipitation was performed as described previously (2). The precipitated proteins were resolved on discontinuous SDS–14% polyacrylamide gels. The 86-amino-acid (aa) and 72-amino-acid forms of Tat are indicated on the left. (C) Function of the various Rex mutant proteins on the heterologous HIV-1 RRE sequence. COS cells were cotransfected with 0.2 μg of the Tat indicator plasmid gTat and 0.1 μg of the indicated DNAs. Tat-specific immunoprecipitation analysis was performed as described for panel B.

tion capacity to determine the functionally important domains in the HTLV-I Rex protein. The results obtained indicated that the mutant Rex1Δ2, which is inactive on the HIV-1 RRE sequence (Fig. 2C, lane 4), is not part of a functional Rex domain. The mutation might affect the tertiary structure of the protein and hence render it incapable of forming the functional domain required for RRE interaction, which is located elsewhere in the Rex protein. The restoration of Rex activity by mutant Rex1Δ3 (Fig. 2C, lane 5), in which the M2 region is completely deleted, supports this hypothesis.

Our results clearly demonstrate that amino-terminal internal deletions of Rex between amino acid residue 35 (mutation M3, Fig. 1) and amino acid residue 55 (mutation M6, Fig. 1) can be tolerated with respect to function on the RxRE and RRE sequences (Fig. 2B, lanes 4 to 8; Fig. 2C, lanes 5 to 8). Further internal deletions, as in mutant Rex1Δ7, resulted in reduced activity on the RxRE sequence and loss of activity on the RRE sequence (Fig. 2B and C, lane 9). Larger internal deletions (Rex1Δ8 to Rex1Δ11) abolished the *trans*-

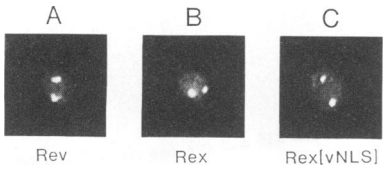

FIG. 3. Subcellular localization of Rev and Rex proteins visualized by indirect immunofluorescence. COS7 cell cultures were transfected with the indicated DNAs and subjected to immunofluorescence with the rabbit polyclonal anti-Rev antiserum Rev1/20 (5) (A) and the rabbit polyclonal anti-Rex antiserum Rex173/189 (B and C).

acting phenotype completely on both target sequences (Fig. 2B and C, lanes 10 to 13).

The constructs with internal deletions at the carboxy-terminal end of the Rex protein displayed an almost identical phenotype on both target sequences. The mutant encoded by Rex20Δ28 represents the border of the domain required for Rex action. Internal deletions extending more amino-terminal of Rex amino acid residue 133 (mutation M20, Fig. 1) were inactive on both sequence elements. In conclusion, our data indicate that a Rex protein core domain, located between amino acid residues 55 and 132, is required for Rex function on the RxRE and RRE sequences.

Concentration in the nucleolar compartment of HTLV-I Rex and HIV-1 Rev is required for *trans* activation of both proteins (3, 26, 37). This nucleolar localization is dependent on the presence of amino acid sequences rich in arginine residues, which constitute the nuclear localization signal (3, 21, 23, 27, 35). We substituted the Rex wild-type nuclear localization signal (Rex amino acid residues 1 to 19 [35]) with the HIV-1 sequence (Rev amino acid residues 33 to 46), generating the *rev-rex* hybrid plasmid Rex[vNLS] (Fig. 1, Table 1). Indirect immunofluorescence was unable to detect any difference in the subcellular accumulation of the wild-type Rev, the wild-type Rex, or the hybrid Rex[vNLS] protein (Fig. 3). Therefore, this result indicated that subregions in the Rev (amino acid residues 33 to 46; Fig. 1) and Rex (amino acid residues 2 to 21; Fig. 1) proteins confer localization to the identical subcellular compartment.

A recent study on bacteriophage antiterminator proteins showed that the interaction of these proteins with RNA hairpins is mediated by arginine-rich motifs (22). Strikingly, the nuclear localization signals of the HIV-1 Rev and HTLV-I Rex proteins also include a cluster of arginine amino acid residues. Therefore, it is speculated that the nuclear localization signal also serves as a response element-binding domain (23). However, the hybrid protein encoded by Rex[vNLS] was active on the RxRE sequence (Table 1). This suggests that, in the Rex protein, regions other than the Rex nuclear localization signal contribute to Rex-specific RxRE binding. Clearly, our experiments cannot rule out the possibility that the wild-type Rex nuclear localization signal represents part of the RNA-binding domain. Due to its positive charge, the nuclear localization signal may facilitate the binding event. However, substitution of the Rev nuclear localization signal in the HIV-1 Rev protein by homologous Rex sequences failed to confer RxRE binding activity (data not shown).

Further mutagenesis and development of in vivo and in vitro Rex assay systems will allow the precise mapping of protein domains required for Rex action. This will help to understand the molecular mechanism of HTLV-I Rex and HIV-1 Rev function more precisely. In addition, both viral *trans*-activator proteins will be helpful tools in the understanding of cellular mRNA processing mechanisms.

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