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Dominant Negative Mutants of Human T-Cell Leukemia Virus Type I Rex and Human Immunodeficiency Virus Type 1 Rev Fail To Multimerize In Vivo

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Human T-cell leukemia virus type I (HTLV-I) Rex and human immunodeficiency virus type 1 (HIV-1) Rev are essential gene products required for the replication of these two pathogenic human retroviruses. Both Rex and Rev act at a posttranscriptional level by binding to highly structured RNA-response elements, the Rex-response element in HTLV-I and the Rev-response element in HIV-1. Using a sensitive in vivo assay of protein-protein interaction, we now demonstrate that the HTLV-I Rex and HIV-1 Rev proteins readily form homomultimeric complexes in the absence of their cognate RNA-response elements yet fail to form heteromultimeric complexes with each other. Dominant negative mutations have been identified in both the *rex* and *rev* genes which presumably specify a critical activation or effector domain in each of these viral transactivators. Surprisingly, these dominant negative mutants of Rex and Rev fail to interact in vivo. These findings raise the possibility that the binding of nonfunctional monomers rather than functional multimers underlies the transdominant phenotype of these Rex and Rev mutants. Further, it seems likely that the assembly of functional and stable multimers of Rex and Rev in vivo may depend not only on the intrinsic multimerization domains of these proteins but also on the binding of a bridging cellular cofactor to the related activation domains present in each viral transactivator.

Type I human T-cell leukemia virus (HTLV-I) and type 1 human immunodeficiency virus (HIV-1) correspond to pathogenic human retroviruses that produce distinctly different clinical disease states. HTLV-I corresponds to the etiologic agent in the adult T-cell leukemia, a neoplastic proliferation of mature and activated $CD4^+$ T lymphocytes (38, 51, 56). In addition, HTLV-I has recently been linked with tropical spastic paraparesis or HTLV-I-associated myelopathy, a progressive demyelinating neuropathic syndrome bearing a superficial resemblance to multiple sclerosis (14, 37). In contrast, HIV-1 infection is associated with the fatal condition AIDS, which is characterized by progressive $CD4^+$ T-cell death rather than T-cell transformation (4, 10, 13). Although HTLV-I is a type C oncogenic retrovirus while HIV-1 is the prototypical human member of the lentivirus family, both of these viruses utilize strikingly similar strategies for the regulated expression of their structural (*gag* and *env*) and enzymatic (*pol*) gene products. Specifically, both viruses encode essential nuclear *trans*-regulatory proteins, Rex in HTLV-I and Rev in HIV-1, that act posttranscriptionally to promote cytoplasmic expression and translation of the unspliced and singly spliced species of viral mRNAs that uniquely encode the *gag-pol* and *env* gene products, respectively (for reviews, see references 17 and 41 and references therein). In the absence of Rex and Rev, these viral mRNAs remain sequestered in the nucleus, where they are either degraded or completely spliced to yield a physically smaller class of viral mRNAs that encode the various regulatory gene products (*tax* and *rex* in HTLV-I or *tat*, *rev*, and *nef* in HIV-1) (20, 25, 29). Rex and Rev exert their functional effects by directly binding to highly structured, but unrelated, RNA response elements (1, 3, 5, 8, 15, 20, 21, 32, 52, 55, 57). The Rex-response element (RexRE) is located in the 3′ long terminal repeat of HTLV-I (20, 45, 49), while the Rev-response element (RevRE) is positioned within the *env* coding region of HIV-1 (9, 18, 30, 42). Surprisingly, despite their general lack of sequence homology, the HTLV-I Rex protein is capable of functionally replacing Rev in the HIV-1 replicative life cycle (11, 40). However, Rex has been shown to bind to the HIV-1 RevRE at a high-affinity site distinct from that recognized by Rev (5, 48). In contrast, HIV-1 Rev fails to replace the function of Rex, indicating a nonreciprocal pattern of genetic complementation (1, 11, 40).

The precise biochemical mechanism underlying the biological effects of Rex and Rev remains unknown. These proteins have been proposed to act either by inhibiting spliceosome function (6), thereby allowing nuclear export of the unspliced or singly spliced viral mRNAs by default, or alternatively by selectively activating a nuclear transport pathway that promotes cytoplasmic delivery and translation of these incompletely processed viral mRNAs (18, 19, 30). Functional analyses of an extensive series of missense mutations within both the HTLV-I *rex* and HIV-1 *rev* genes have revealed the presence of at least two functional domains in both proteins (27, 31, 39, 53). One domain corresponds to an arginine-rich motif that mediates RNA binding and nuclear localization. Mutations within this domain yield recessive negative mutants of Rex and Rev that both lack biological activity and when coexpressed fail to interfere with the function of their wild-type counterparts. A second, more C-terminal domain corresponds to a presumed activation or effector region which when mutated leads to a dominant negative class of mutants that not only lack biological activity but also interfere with the function of the

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wild-type transactivator. As expected, these dominant negative mutants of Rex and Rev retain full capacity to specifically engage their RNA response elements, as their arginine-rich RNA binding domains are unaltered (5, 28).

Previous studies have suggested the presence of yet a third domain in the HIV-1 Rev protein that mediates multimerization of this transactivator protein (27, 28, 36, 58). This domain immediately flanks the arginine-rich motif, and mutations therein have been shown to lead to a loss of Rev function with variably either a recessive negative or a weakly dominant negative functional phenotype (28). However, the precise role that protein-protein interaction plays in the in vivo biological action of both Rex and Rev remains incompletely understood. In this study, we have used an in vivo assay of protein-protein association, modified from that first described by Fields and Song in yeast cells (12), to study the ability of the HTLV-I Rex and HIV-1 Rev proteins to form homo- and heteromultimeric complexes in living cells in the absence of their cognate RNA response elements. We now demonstrate that Rex and Rev readily form homomultimers in vivo yet fail to assemble as heteromultimers with each other. We further show that dominant negative mutations within both Rex and Rev completely abrogate this in vivo protein-protein association. Together, these findings suggest that these protein-protein interactions are inextricably linked with the biological function of Rex and Rev and that one mechanism of transdominant inactivation may involve the blockade of these critical protein-protein interactions.

MATERIALS AND METHODS

In vivo assay of protein-protein interaction. The ability of the HTLV-I Rex and HIV-1 Rev proteins to associate in vivo was studied by using a modified version of the yeast GAL4-based assay first described by Fields and Song (12). In brief, Rex and Rev cDNAs were fused to either the DNA binding domain of GAL4 (residues 1 to 147) (44) or the strong transcriptional activation domain of the herpes simplex virus-derived VP16 gene product (residues 413 to 490) (50). These various expression vectors were then transfected into COS cells, either alone or in combination but always in the presence of the chloramphenicol acetyltransferase (CAT) reporter plasmid pG_5BCAT, containing five binding sites for GAL4 (43). Effective protein-protein interaction by either Rex or Rev is predicted to spatially juxtapose the GAL4 DNA binding domain and VP16 transcriptional activation domain encoded by the different plasmids, which in turn leads to stimulation of the pG_5BCAT reporter plasmid.

GAL4 chimeric expression plasmids. To produce the various chimeric GAL4 expression vectors, the coding sequences of Rex and Rev were amplified by the polymerase chain reaction and ligated in frame downstream of the first 147 codons of the yeast GAL4 transcription factor contained within the pSG424 expression vector (44). In addition to the entire coding sequence of Rex (amino acids 1 to 189), three N-terminal deletions of Rex (amino acids 32 to 189, 51 to 189, and 71 to 189) were ligated as *Bam*HI-*Sac*I fragments into plasmid pSG424 to produce the GAL4-Rex(32-189), GAL4-Rex(51-189), and GAL4-Rex(71-189) expression vectors. Similarly, the complete coding sequence of Rev (codons 1 to 116) and three missense mutations of Rev, M4 (28), M28, and M33 (27) (see Table 2), were ligated as *Kpn*I-*Sac*I fragments into the pSG424 vector.

VP16 chimeric expression plasmids. To produce the second set of chimeric VP16 expression vectors, the coding sequence of the 78-residue acidic activation domain of the herpes simplex virus VP16 gene supplemented with a C-terminal stop codon (43, 50) was ligated in frame into unique extreme 3′ *Bgl*II sites of pcRex M18 (39), pcRev M14 (27), and pc TaxM52 (47). Each of these mutations is located very near the C terminus of the natural protein, and each mutant retains wild-type biological activity.

TABLE 1. CAT activities of Rex mutants

Rex protein	Altered amino acids	Phenotype	CAT activity[a] (% of WT)
WT		WT	100
M4	33–35 (DTQ)	WT	113.5 ± 7.0
M5	43, 44 (YK)	WT	86.9 ± 9.4
M6	59, 60 (YI)	DN[b]	11.6 ± 2.7
M7	64, 65 (YW)	DN	19.8 ± 3.8
M8	72, 73 (RS)	WT	97.0 ± 17.5
M10	101, 102 (EP)	WT	100.0 ± 17.2
M13	119–121 (TFH)	DN	10.5 ± 4.3
M14	134–136 (SEM)	WT	107.4 ± 16.5

[a] Dominant negative HTLV-I Rex mutants fail to multimerize in vivo. The GAL4-Rex and Rex (missense or wild type [WT])-VP16 expression vectors were cotransfected into COS cells with the pG_5BCAT reporter plasmid and assayed for induction of CAT enzyme activity. The level of CAT expression obtained with cotransfection of combinations of GAL4-Rex and Rex-VP16 was assigned a value of 100%. Rex mutants M4, M5, M8, M10, and M14 retain wild-type biological activity, while the M6, M7, and M13 mutants correspond to dominant negative mutants (39). Data presented correspond to the mean levels ± standard errors of the means obtained from three independent transfection experiments.

[b] DN, dominant negative.

In addition to the pcRex-VP16 expression vector which encodes the first 181 amino acids of Rex followed by the 78-residue activation domain of VP16, a series of C-terminal deletions of Rex was fused to the VP16 activation domain, thus generating the pcRex(1-118)-VP16, pcRex(1-133)-VP16, and pcRex(1-161)-VP16 expression vectors, respectively. The pcRex(missense)-VP16 expression vectors were constructed by excision of a wild-type DNA fragment (*Hin*dIII-*Cla*I or *Cla*I-*Bgl*II) from the Rex-VP16 expression vector, followed by insertion of the equivalent but mutated DNA from the previously described Rex analog, M4, M5, M6, M7, M8, M10, M13, and M14 (39) (Table 1). The pcRev-VP16 vector encodes the initial 111 amino acids of Rev followed by the activation domain of VP16, while the pcTax-VP16 vector encodes the first 351 amino acids of the HTLV-1 Tax protein followed by the activation domain of VP16.

Cell transfections. COS monkey kidney cells were cotransfected with 2 μg of CAT reporter plasmid pG_5BCAT, 4 μg of GAL4 chimeric expression plasmid, and 4 μg of VP16 chimeric expression plasmid, using DEAE-dextran followed by incubation with chloroquine and dimethylsulfoxide shock (46). As needed, pCMV-IL2 plasmid DNA (7) was added to maintain a constant DNA concentration in all transfections. Approximately 48 h after transfection, cell extracts were prepared and tested for CAT activity, using the phase separation assay described by Neumann et al. (35).

Western immunoblotting of chimeric proteins. To confirm production of protein by each expression vector, Western blotting studies were performed with COS cells transfected with 4 μg of each plasmid DNA; 48 h later, the cultures were lysed in radioimmunoprecipitation buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 8.0]) (29), and the extract proteins were separated on SDS–10% polyacrylamide gels. The proteins were then transferred to nitrocellulose filters

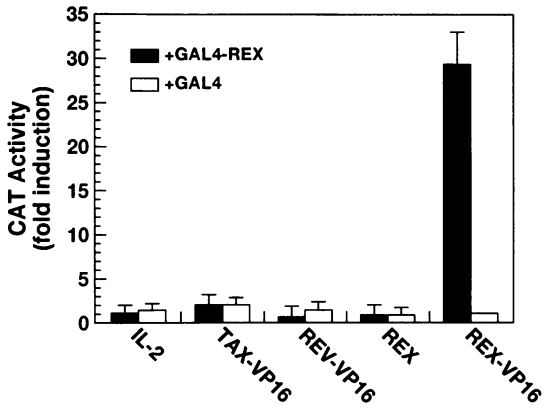

FIG. 1. Evidence that the HTLV-I Rex protein forms apparent homomultimeric complexes in vivo in the absence of the RexRE. COS cells were cotransfected with various combinations of the GAL4 and VP16 chimeric expression vectors as indicated and pG_5BCAT, and the cultures were assayed for CAT enzyme activity 48 h later. The level of CAT activity resulting from the coexpression of GAL4 and Rex-VP16 was used as the baseline. Data presented correspond to the mean fold stimulation levels ± standard errors of the means obtained in five independent transfection experiments.

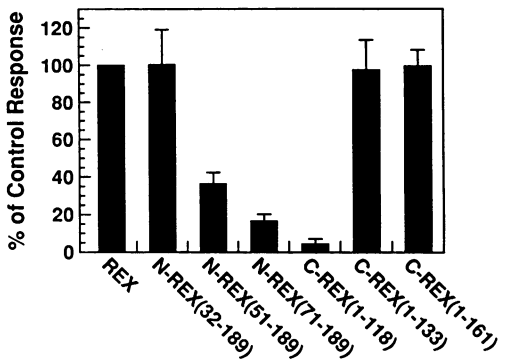

FIG. 2. Evidence that sequences required for in vivo multimerization of the HTLV-I Rex protein are located between residues 32 and 133. Each of the chimeric Rex deletion expression vectors was designed such that the resultant protein contained a functional nuclear localization signal. The N-terminal deletion constructs were fused to GAL4, allowing utilization of the GAL4 nuclear localization signal, while the Rex C-terminal deletions were fused to the VP16 activation and thus utilized the native Rex nuclear localization signal. COS cells were cotransfected with either GAL4-Rex and Rex(C-terminal deletion)-VP16 or GAL4-Rex(N-terminal deletion) and Rex-VP16. The level of transactivation resulting from the coexpression of GAL4-Rex and Rex-VP16 was assigned a value of 100%. Data presented correspond to the mean fold stimulation levels ± standard errors of the means obtained from three independent transfection experiments.

and incubated with either specific anti-Rex (39), anti-Rev (27), or anti-Tax (47) antisera (1:1,000 dilution) followed by ^{125}I-protein A. Immunoreactive proteins were then visualized by autoradiography.

RESULTS

As shown in Fig. 1, expression of the VP16-Rex vector alone or in conjunction with the GAL4 expression vector produced no significant activation of the pG_5BCAT reporter plasmid. However, coexpression of the Rex-VP16 and GAL4-Rex plasmids led to nearly a 30-fold increase in CAT activity. This transcriptional activation was dependent on the presence of both the GAL4 DNA binding domain and VP16 activation domain, as cotransfection of vectors encoding Rex and Rex-VP16 or GAL4-Rex and Rex failed to produce significant stimulation (Fig. 1). The observed increases in CAT expression also reflected a specific Rex-Rex protein interaction, as coexpression of GAL4-Rex with either Rev-VP16 or HTLV-I Tax-VP16 produced no significant stimulation. Of note, the Rex-Rex interaction occurring in *trans* appeared quite stable, as the level of CAT activity obtained was consistently greater than one-third of that measured with the GAL4-VP16 vector, which links in *cis* the DNA binding domain of GAL4 and the transcriptional activation domain of VP16 (43). Similar stimulatory effects were obtained when the transcriptional activation domain of NF-κBp65 (2) was substituted for the VP16 component, indicating the general nature of these stimulatory effects.

To identify sequences within Rex required for this in vivo protein-protein interaction, a series of expression vectors encoding various N- and C-terminal deletions was tested (Fig. 2). These vectors were specifically designed to ensure that each chimeric protein retained a functional nuclear localization signal. A chimeric protein composed of Rex residues 32 to 189 was found to retain full multimerization activity (Fig. 2). In contrast, deletion of 50 or 70 N-terminal residues of Rex [N-Rex(51-189) or N-Rex(71-189)] produced a partial or nearly complete loss of in vivo multimerization activity (Fig. 2). Similar analyses of the C-terminal deletion series revealed that the final 56 amino acids of Rex [C-Rex(1-133)] were entirely dispensable for in vivo assembly, while deletion of 15 additional carboxy amino acids [C-Rex(1-118)] markedly attenuated this protein-protein interaction (Fig. 2). These studies thus mapped the extreme boundaries of the Rex domain(s) required for in vivo protein-protein association to a segment residing between residues 32 and 133.

Strikingly, the boundaries of this domain involved in vivo multimerization of Rex appeared coincident with those of a domain of Rex required for its biological activity (22). To further investigate this finding, a series of previously characterized Rex missense mutations within this domain (39) was tested in the context of the VP16-Rex expression vector. These missense mutants included three dominant negative mutations that inactivated this Rex effector domain as well as five control point mutations scattered throughout this region that failed to alter the resultant biological activity of Rex. Each of the three dominant negative point mutants of Rex displayed markedly reduced capacity to associate in vivo, while each of the five missense mutants retaining wild-type biological activity displayed normal activity (Table 1). Importantly, each of these inactive dominant-negative Rex fusion proteins was expressed in a stable manner, as assessed by Western blotting (Fig. 3).

In vivo protein-protein interaction of the HIV-1 Rev protein was similarly studied (Fig. 4). Coexpression of GAL4-Rev and Rev-VP16 vectors induced approximately an eightfold activation of CAT activity compared with the various negative controls. As with Rex, this response required coexpression of both the GAL4 DNA binding domain and the VP16 activation domain, each linked in *cis* to Rev. No response was detected following expression of the DNA binding domain of GAL4 with Rev-VP16 or the GAL4-Rev plasmid alone (Fig. 4). Further, no heteromultimerization of Rev with Rex or Tax was observed. Of note, CAT activity induced by GAL-4 Rev and Rev-VP16 combinations was

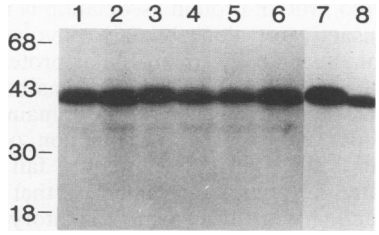

FIG. 3. Western blotting analysis of the Rex-VP16 fusion protein expression in COS cells. Shown are results for Rex M4 (lane 1), Rex M5 (lane 2), Rex M6 (lane 3), Rex M7 (lane 4), Rex M8 (lane 5), Rex M10 (lane 6), Rex M13 (lane 7), and Rex M14 (lane 8). Functional results for these Rex mutants are presented in Table 1. Sizes are indicated in kilodaltons.

consistently only 25 to 30% of the response obtained with GAL-4 Rex and Rex-VP16 and only 8 to 10% of the response obtained with the GAL4-VP16 plasmid, in which the two domains are linked in *cis*.

To determine whether the previously described activation and multimerization domains of Rev were required for this in vivo protein-protein interaction, three previously described missense mutations of Rev were tested as fusion proteins with the DNA binding domain of GAL4 (Table 2) (27, 28, 31). Both wild-type Rev and a Rev missense mutant (M33) altered near but not within the leucine-rich activation domain readily interacted in vivo. In sharp contrast, a GAL4-Rev chimera containing the dominant negative M28 mutation altered within the leucine-rich activation domain of Rev failed to associate in vivo. Further, a three-residue mutation of Rev (M4) within the more N-terminal component of its multimerization domain (28) (amino acids residues 23 to 31 and 54 to 57) also produced diminished CAT expression. Despite their lack of functional activity in this assay, each of these mutants was stably expressed, as assessed by Western blotting with Rev-specific antisera (Fig. 5).

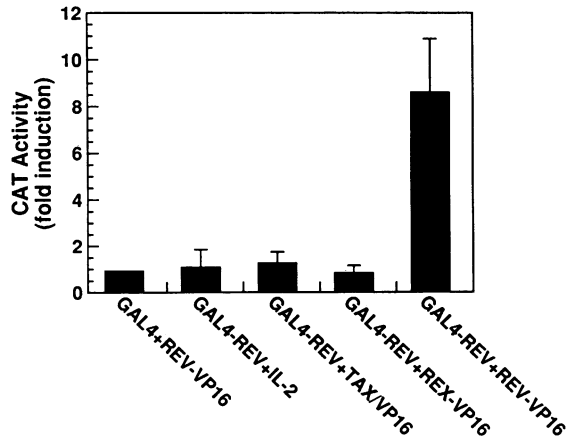

FIG. 4. Evidence that the HIV-I Rev protein forms apparent homomultimeric complexes in vivo in the absence of the RevRE. COS cells were cotransfected with various combinations of the GAL4 and VP16 chimeric expression plasmids and pG_5BCAT reporter plasmid as indicated. The level of transactivation resulting from the coexpression of GAL4 and Rev-VP16 was used as background. Data presented correspond to the mean fold stimulation levels ± standard errors of the means derived from three independent transfection experiments.

TABLE 2. CAT activities of Rev mutants

Rev protein	Change from WT			Phenotype	CAT activity[a] (% of WT)
	WT	Altered amino acids	Mutation		
WT		None		WT	100
M4	YQSN	23, 25, 26	DQDL	DN[b] (weak)	6.7 ± 2.5
M28	L	81	A	DN	0.6 ± 0.6
M33	DC	84, 85	NS	WT	98.0 ± 9.6

[a] Dominant negative HIV-I Rev mutants fail to multimerize in vivo. The GAL4-Rev (missense and wild type [WT]) and Rev-VP16 expression vectors were cotransfected into COS cells with the pG_5BCAT reporter plasmid and assayed for the induction of CAT enzyme activity. The level of CAT expression obtained with combinations of the GAL4-Rev and Rev-VP16 plasmids was assigned a value of 100%. Rev mutant M33 retains wild-type biological activity, while the M4 multimerization domain mutant and the M28 leucine-rich activation domain mutant displayed weak and strong recessive negative and dominant negative phenotypes, respectively (27, 28, 31). Data presented correspond to the mean levels ± standard errors of the means obtained from three independent transfection experiments.

[b] DN, dominant negative.

DISCUSSION

The HTLV-I Rex and HIV-1 Rev proteins subserve functionally similar roles in the replicative life cycles of these two human retroviruses. Specifically, both of these essential regulatory proteins promote cytoplasmic expression and translation of the incompletely spliced family viral mRNAs that uniquely encode the structural and enzymatic proteins needed for the assembly of infectious virions (reviewed in references 17 and 41). Further, the action of each of these proteins appears dependent upon direct binding to highly structured RNA response elements (1, 3, 5, 8, 15, 20, 21, 32, 52, 55, 57). Mutational analyses of these *trans*-acting viral polypeptides have further revealed the presence of functionally analogous subregion domains, including an arginine-rich motif mediating RNA binding and nuclear/nucleolar localization and more C-terminal sequences comprising a putative activation domain (27, 31, 39, 53). Indeed, a leucine-rich segment within this activation domain appears functionally interchangeable between Rex and Rev (23, 54). A bipartite multimerization domain located between amino acids 23 to 31 and 54 to 57, segments which immediately flank the arginine-rich motif, has also been described in Rev (28). Mutations in the arginine-rich domains of these transactivators lead to a recessive negative class of mutants that lack the ability to bind to the RNA response elements. In

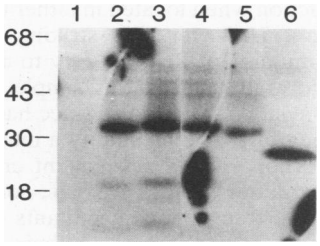

FIG. 5. Western blotting analysis of GAL4-Rev wild-type and mutant protein expression in COS cells. Shown are results for GAL4-Rex (lane 1), GAL4-Rev (lane 2), GAL4-Rev M4 (lane 3), GAL4-Rev M28 (lane 4), GAL4-Rev M33 (lane 5), and Rev-VP16 (lane 6). Functional results of protein-protein interactions with each of these various expression vectors are shown in Table 2. Sizes are indicated in kilodaltons.

contrast, mutations in the more C-terminal leucine-rich activation domain result in dominant negative mutant proteins that retain full RNA binding activity (5, 23, 32). Mutations within the multimerization domain of Rev have been variably described as producing either recessive negative or weakly dominant negative phenotypes (27, 28, 36, 58). An analogous multimerization domain in Rex has not yet been precisely identified, but this domain could well involve residues inactivated by the M6 and M7 dominant negative Rex mutations which are located N terminal of the exchangeable leucine-rich activation domain. At present, the precise role for multimerization of Rex or Rev in their biological function remains unknown. However, it seems likely that these protein-protein interactions may allow the assembly of multiple Rex or Rev molecules on the RNA response element which may be essential for interactions with cellular cofactors needed for Rex and Rev function.

In this study, we demonstrate that both the HTLV-I Rex and HIV-1 Rev proteins are capable of associating in living cells to form apparent homomultimeric complexes in the absence of their cognate RNA response elements. Further, we show that these transactivators are unable to cross-couple in vivo to yield stable Rex-Rev heteromultimeric complexes. Of note, the in vivo association of Rev as measured in this CAT-based transcriptional assay was consistently weaker than that detected with Rex. At present, the stoichiometric composition of the biologically active complexes of Rex and Rev remains unclear, although native Rev has been reported to exist as a tetramer in solution (34, 58). Strikingly, these in vivo protein-protein interactions of both Rex and Rev were consistently disrupted by dominant negative point mutations involving either the multimerization or activation domains of these two viral transactivators. Together, these findings suggest the intriguing possibility that both the activation and dimerization domains of Rex and Rev are importantly involved in protein-protein interactions occurring in vivo and further that these interactions are intrinsically linked to the biological function of these transactivators. Unfortunately, recessive negative mutants of Rex and Rev altered in their arginine-rich RNA binding domains could not be tested in this assay because these chimeric proteins proved consistently unstable when expressed in COS cells.

Dominant negative repressor gene products have been identified in several diverse genetic systems (16, 26, 50). One mechanism leading to such transdominance involves the assembly of inactive mixed multimers, as illustrated by the dominant negative I^{-d} mutants of the prokaryotic *lac* repressor (33). Indeed, such a model has been proposed for transdominant Rev mutants because of their ability to inhibit wild-type Rev action when located in either the cytoplasm or the nucleus (24). However, the striking inability of the transdominant mutants of Rex and Rev to associate in vivo would appear to argue against this simple model. Alternatively, in other systems, transdominance has been shown to reflect occupancy of a binding site by a mutant protein that is incapable of supporting a subsequent critical functional step. In this regard, our findings are quite consistent with the possibility that the transdominant mutants of Rex and Rev bind as nonfunctional monomers to their target RNA sequences in vivo but are perhaps incapable of undergoing either a prior or subsequent protein assembly reaction obligately required for their biological function. Our findings further suggest that the activation domain is intimately involved in these in vivo protein-protein interactions. In this regard, it is particularly intriguing to consider the possibility that this in vivo protein-protein association is not limited to the viral transactivator alone but also involves a bridging cellular factor. Specifically, this cellular protein might represent the long-elusive host factor that has been proposed to bind to the leucine-rich activation domain of Rex and Rev and to be required for the function of these viral proteins. Our finding that Rex and Rev fail to associate heteromerically in vivo could indicate that the cellular factors interacting with these viral regulatory proteins are distinct. However, particularly in view of their fully exchangeable leucine-rich activation domains (23, 54), we suspect that the same cellular factor is involved and that heteromers fail to form because yet a second structural motif, the multimerization domain, is required and fails to support such in vivo cross-coupling of Rex and Rev polypeptides. Indeed, the importance of the multimerization domain in this in vivo assembly reaction is highlighted by the inhibitory effects of the M4 mutation in Rev and by the M6 and M7 mutations in Rex which may specify the multimerization domains in these two transactivators. The apparent lack of effects of the Rev M7 mutation in this assay which has been proposed to specify a downstream portion of a bipartite multimerization domain in Rev brings into question the true function of this segment of Rev. At present, the precise role for this apparent in vivo multimerization reaction of Rex and Rev in the induction of cytoplasmic *gag-pol* and *env* mRNA expression remains poorly understood. However, the near complete failure of the various transdominant mutants of both Rex and Rev to allow such assembly reactions to occur in vivo argues that this process is likely central to the biological action of both of these viral transactivators. Indeed, the binding of inactive monomers of Rex or Rev or perhaps in vivo unstable multimers of Rex or Rev lacking the critical cellular cofactor or even single Rex or Rev molecules linked to the cellular cofactor but not other Rex or Rev molecules could underlie the transdominant phenotype of these various mutants. The prior finding that transdominant mutants of Rev are capable of multimerizing on the RevRE in vitro (28) may underestimate the dynamics and restrictions of the protein-protein interactions occurring in vivo. Alternatively, binding of Rex and Rev to their cognate RNA response elements may serve to further stabilize their interaction, making the cellular factor bound to the activation domain less important. Indeed, binding to RNA could even result in the release of one cellular factor followed by the binding of a different factor at the activation domain, providing an additional level of regulation. Notwithstanding these uncertainties, all of these models argue that Rex and Rev biological action critically involves the recruitment of multiple Rex or Rev molecules and cellular factors to the RNA response element in vivo.

REFERENCES

1. **Ahmed, Y. F., S. M. Hanly, M. H. Malim, B. R. Cullen, and W. C. Greene.** 1990. Structure-function analyses of the HTLV-I Rex and HIV-1 Rev RNA response elements: insights into the mechanism of Rex and Rev action. Genes Dev. **4**:1014–1022.
2. **Ballard, D. W., E. P. Dixon, N. J. Peffer, H. Bogerd, S. Doerre, B. Stein, and W. C. Greene.** 1992. The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc. Natl. Acad. Sci. USA **89**:1875–1879.
3. **Ballaun, C., G. K. Farrington, M. Dobrovnik, J. Rusche, J. Hauber, and E. Böhnlein.** 1991. Functional analysis of human T-cell leukemia virus type I *rex*-response element: direct RNA binding of Rex protein correlates with in vivo activity. J. Virol. **65**:4408–4413.

4. **Barré-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamarel, T. Gruest, C. Davguet, C. Axler-Blin, F. Vezin-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphocyte retrovirus from a patient at risk for acquired immune deficiency syndrome. Science **220:**868–870.
5. **Bogerd, H. P., G. L. Huckaby, Y. F. Ahmed, S. M. Hanly, and W. C. Greene.** 1991. The type I human T-cell leukemia virus (HTLV-I) Rex trans-activator binds directly to the HTLV-I Rex and type I human immunodeficiency virus Rev RNA response elements. Proc. Natl. Acad. Sci. USA **88:**5704–5708.
6. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of RNA splice sites. Cell **59:**789–795.
7. **Cullen, B. R.** 1986. Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46:**973–982.
8. **Daly, T. J., K. S. Cook, G. S. Gray, T. E. Malone, and J. R. Rusche.** 1989. Specific binding of the HIV-1 recombinant Rev protein to the Rev-responsive element *in vitro*. Nature (London) **342:**816–819.
9. **Dayton, E., D. Powell, and A. Dayton.** 1989. Functional analysis of CAR, the target sequence for the Rev protein of HIV-1. Science **246:**1625–1629.
10. **Fauci, A. S.** 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science **239:**617–622.
11. **Felber, B. K., D. Derse, A. Athanassopoulos, M. Campbell, and G. N. Pavlakis.** 1989. Cross-activation of the rex proteins of HTLV-1 and BLV and of the Rev protein of HIV-1 and nonreciprocal interactions with their RNA responsive elements. New Biol. **1:**318–330.
12. **Fields, S., and O.-K. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
13. **Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Sasai, G. White, P. Foster, and P. D. Markham.** 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk of AIDS. Science **224:**500–503.
14. **Gessain, A., F. Barin, J. C. Vernant, O. Gout, L. Maurs, A. Calender, and G. de The.** 1985. Antibodies to the human T lymphotropic virus type-1 in patients with tropical spastic paraparesis. Lancet **ii:**407–410.
15. **Grassmann, R. S., S. Berchtold, C. Aepinus, C. Ballaun, E. Böhnlein, and B. Fleckenstein.** 1991. In vitro binding of human T-cell leukemia virus *rex* proteins to the *rex*-response element of viral transcripts. J. Virol. **65:**3721–3727.
16. **Glenn, G. M., and R. P. Ricciardi.** 1987. An adenovirus type 5 E1A protein with a single amino acid substitution blocks wild-type E1A transactivation. Mol. Cell. Biol. **7:**1004–1011.
17. **Greene, W. C., and B. R. Cullen.** 1990. The Rev-Rex connection: convergent strategies for the post-transcriptional regulation of HIV-1 and HTLV-I gene expression. Semin. Virol. **1:**195–204.
18. **Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Toe, and G. N. Pavlakis.** 1989. The *rev* (*trs/art*) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a *cis*-acting sequence in the *env* region. J. Virol. **63:**1265–1274.
19. **Hammarskjold, M. L., J. Heimer, B. Hammarskjold, I. Sangwan, and A. L. Rekosh.** 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. J. Virol. **63:**1959–1966.
20. **Hanly, S. M., L. T. Rimsky, M. H. Malim, J. H. Kim, J. Hauber, M. DucDodon, S.-Y. Le, J. V. Maizel, B. R. Cullen, and W. C. Greene.** 1989. Comparative analysis of the HTLV-I Rex and HIV-1 Rev trans-regulatory proteins and their RNA response elements. Genes Dev. **3:**1534–1544.
21. **Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. M. Green, J. Karn, A. D. Louie, M. Singh, and M. A. Skinner.** 1990. HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. Cell **60:**685–693.
22. **Hofer, L., I. Weichselbraun, S. Quick, G. K. Farrington, E. Böhnlein, and J. Hauber.** 1991. Mutational analysis of the human T-cell leukemia virus type I *trans*-acting *rex* gene product. J. Virol. **65:**3379–3383.
23. **Hope, T. J., B. L. Bond, D. McDonald, N. P. Klein, and T. G. Parslow.** 1991. Effector domains of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex are functionally interchangeable and share an essential peptide motif. J. Virol. **65:**6001–6007.
24. **Hope, T. J., N. P. Klein, M. E. Elder, and T. G. Parslow.** 1992. *trans*-dominant inhibition of human immunodeficiency virus type 1 Rev occurs through formation of inactive protein complexes. J. Virol. **66:**1849–1855.
25. **Inoue, J. I., M. Yoshida, and M. Seiki.** 1987. Transcriptional ($p40^{x}$) and post-transcriptional ($p27^{x\text{-}III}$) regulators are required for the expression and replication of human T-cell leukemia virus type I genes. Proc. Natl. Acad. Sci. USA **84:**3653–3657.
26. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37:**801–813.
27. **Malim, M. H., S. Böhnlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 Rev trans-activator-derivation of a trans-dominant repressor of Rev function. Cell **58:**205–214.
28. **Malim, M. H., and B. R. Cullen.** 1991. HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. Cell **65:**241–248.
29. **Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen.** 1988. Immunodeficiency virus rev trans-activator modulates expression of the viral regulatory genes. Nature (London) **335:**181–183.
30. **Malim, M. H., J. Hauber, S.-Y. Lee, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338:**254–257.
31. **Malim, M. H., D. F. McCarn, L. S. Tiley, and B. R. Cullen.** 1991. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J. Virol. **65:**4248–4254.
32. **Malim, M. H., L. S. Tiley, D. F. McCarn, J. R. Rusche, J. Hauber, and B. R. Cullen.** 1990. HIV-1 structural gene expression requires binding of the Rev trans-activator to its RNA target sequence. Cell **60:**675–683.
33. **Miller, J. H.** 1980. The *lac* I gene: its role in *lac* operon control and its use as a genetic system, p. 31–88. *In* J. H. Miller and W. R. Reznikoff (ed.), The operon, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Nalin, C. M., R. D. Purcell, D. Antelman, D. Mueller, L. Tomchak, B. Wegrzynski, E. McCarney, V. Toome, R. Kramer, and M.-C. Hsu.** 1990. Purification and characterization of recombinant Rev protein of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **87:**7593–7597.
35. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyl transferase gene expression. BioTechniques **5:**444–448.
36. **Olsen, H. S., A. W. Cochrane, P. J. Dillon, C. M. Nalin, and C. A. Rosen.** 1990. Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in *env* mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. Genes Dev. **4:**1357–1364.
37. **Osame, M., K. Usuku, N. Ijichi, H. Amitani, A. Igata, M. Matasumoto, and H. Tara.** 1986. HTLV-I associated myelopathy, a new clinical entity. Lancet **i:**1031–1032.
38. **Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA **77:**7415–7419.
39. **Rimsky, L., M. DucDodon, E. P. Dixon, and W. C. Greene.** 1989. Trans-dominant inactivation of HTLV-I and HIV-1 gene expression by mutation of the HTLV-I Rex transactivator. Nature (London) **341:**453–456.
40. **Rimsky, L., J. Hauber, M. Dukovich, M. H. Malim, A. Langlois, B. R. Cullen, and W. C. Greene.** 1988. Functional replacement of the HIV-1 Rev protein by the HTLV-I Rex protein. Nature (London) **335:**783–740.

41. **Rosen, C. A., and G. N. Pavlakis.** 1990. Tat and Rev: positive regulators of HIV gene expression. AIDS J. **4:**449–509.
42. **Rosen, C. A., E. Terwillinger, A. Dayton, J. G. Sodroski, and W. A. Haseltine.** 1988. Intragenic *cis*-acting art gene-responsive sequences of the human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **85:**2071–2075.
43. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature (London) **335:**563–564.
44. **Sadowski, I., and M. Ptashne.** 1989. A vector for expressing GAL4(1–147) fusions in mammalian cells. Nucleic Acids Res. **17:**7539.
45. **Seiki, M., J.-I. Inoue, M. Hidaka, and M. Yoshida.** 1988. Two cis-acting elements responsible for post-transcriptional trans-regulation of gene expression of human T-cell leukemia virus type 1. Proc. Natl. Acad. Sci. USA **85:**7124–7128.
46. **Selden, R.** 1987. Transfection using DEAE-dextran. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, supplement 14, p. 9.2. John Wiley & Sons, New York.
47. **Smith, M. R., and W. C. Greene.** 1990. Identification of HTLV-1 *tax* trans-activator mutants exhibiting novel transcriptional phenotypes. Genes Dev. **4:**1875–1885.
48. **Solomin, L., B. K. Felber, and G. N. Pavlakis.** 1990. Different sites of interaction for *rev*, *tev*, and *rex* proteins with the *rev*-response element of the human immunodeficiency virus type 1. J. Virol. **64:**6010–6017.
49. **Toyoshima, H., M. Itoh, J. Inoue, M. Seiki, F. Takaku, and M. Yoshida.** 1990. Secondary structure of the human T-cell leukemia virus type 1 *rex*-responsive element is essential for *rex* regulation of RNA processing and transport of unspliced RNAs. J. Virol. **64:**2825–2832.
50. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev. **2:**718–729.
51. **Uchiyama, T., J. Yodoi, K. Sagawa, K. Takatsuki, and H. Uchino.** 1977. Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood **50:**481–491.
52. **Unge, T., L. Solomin, M. Mellini, D. Derse, B. K. Felber, and G. N. Pavlakis.** 1991. The Rex regulatory protein of human T-cell lymphotropic virus type I binds specifically to its target site within the viral RNA. Proc. Natl. Acad. Sci. USA **88:**7145–7149.
53. **Weichselbraun, I., J. Berger, M. Dobrovnik, H. Bogerd, R. Grassmann, W. C. Greene, J. Hauber, and E. Böhnlein.** 1992. Dominant-negative mutants are clustered in a domain of the human T-cell leukemia virus type I Rex protein: implications for *trans*-dominance. J. Virol. **66:**4540–4545.
54. **Weichselbraun, I., G. K. Farrington, J. R. Rusche, E. Böhnlein, and J. Hauber.** 1992. Definition of the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex protein activation domain by functional exchange. J. Virol. **66:**2583–2587.
55. **Yip, M., W. S. Dynan, P. L. Green, A. C. Black, S. Arrigo, A. Torbats, S. Heaphy, C. Ruland, J. D. Rosenblatt, and I. S. Y. Chen.** 1991. Human T-cell leukemia virus (HTLV) type II Rex protein binds specifically to RNA sequences of the HTLV long terminal repeats but poorly to the human immunodeficiency virus type 1 Rev-responsive element. J. Virol. **65:**2261–2272.
56. **Yoshida, M., I. Miyoshi, and Y. Hinuma.** 1982. Isolation and characterization of retrovirus from cell lines of adult T-cell leukemia and its implication in the disease. Proc. Natl. Acad. Sci. USA **79:**2031–2035.
57. **Zapp, M. L., and M. R. Green.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature (London) **342:**714–716.
58. **Zapp, M. L., T. J. Hope, T. G. Parslow, and M. R. Green.** 1991. Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. Proc. Natl. Acad. Sci. USA **88:**7734–7738.