MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4207–4214 Vol. 16, No. 8
0270-7306/96/$04.00+0


# Protein Sequence Requirements for Function of the Human T-Cell Leukemia Virus Type 1 Rex Nuclear Export Signal Delineated by a Novel In Vivo Randomization-Selection Assay

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Received 12 December 1995/Returned for modification 9 April 1996/Accepted 26 April 1996

**The Rex protein of human T-cell leukemia virus type 1, like the functionally equivalent Rev protein of human immunodeficiency virus type 1, contains a leucine-rich activation domain that specifically interacts with the human nucleoporin-like Rab/hRIP cofactor. Here, this Rex sequence is shown to function also as a protein nuclear export signal (NES). Rex sequence libraries containing randomized forms of the activation domain/NES were screened for retention of the ability to bind Rab/hRIP by using the yeast two-hybrid assay. While the selected sequences differed widely in primary sequence, all were functional as Rex activation domains. In contrast, randomized sequences that failed to bind Rab/hRIP lacked Rex activity. The selected sequences included one with homology to the Rev activation domain/NES and a second that was similar to the NES found in the cellular protein kinase inhibitor α. A highly variant, yet fully active, activation domain sequence selected on the basis of Rab/hRIP binding retained full NES function even though this sequence preserved only a single leucine residue. In contrast, nonfunctional activation domain mutants that were unable to bind Rab/hRIP had also lost NES function. These data demonstrate that NES activity is a defining characteristic of the activation domains found in the Rev/Rex class of retroviral regulatory proteins and strongly support the hypothesis that the Rab/hRIP cofactor plays a critical role in mediating the biological activity of these NESs. In addition, these data suggest a consensus sequence for NESs of the Rev/Rex class.**

The pathogenic complex retroviruses human T-cell leukemia virus type 1 (HTLV-1) and human immunodeficiency virus type 1 (HIV-1) belong to distinct retroviral families and display little sequence homology. The approaches used by these viruses to regulate proviral gene expression are nevertheless remarkably similar (reviewed in reference 5). Thus, each virus encodes a transcriptional regulatory protein that acts on the respective viral long terminal repeat promoter element to dramatically enhance viral gene expression. In addition, each virus also encodes an essential posttranscriptional regulatory protein, termed Rex in HTLV-1 and Rev in HIV-1, that induces the sequence-specific nuclear export, and hence translation, of the incompletely spliced viral mRNA species that encode the various viral structural proteins (8, 11, 16, 17, 20, 23, 27).

Initial evidence favoring the hypothesis that Rex and Rev, despite lacking any significant sequence identity, might nevertheless mediate the nuclear export of target RNAs via the same mechanism came from the finding that Rex could partly rescue the replication of a Rev-deficient HIV-1 provirus (36). Subsequently, both Rev and Rex were shown to bind directly to structured RNA response elements present in their target RNAs (3, 6, 14, 19, 29, 38, 41) and to contain multimerization domains that are essential for the recruitment of additional Rev or Rex molecules (Fig. 1) (2, 26, 34). In addition, both Rev and Rex contain short, leucine-rich sequence elements, termed activation domains, that recruit an essential cellular cofactor to the resultant ribonucleoprotein complex (Fig. 1) (21, 25, 28, 39). These activation domains can be reciprocally exchanged between these two proteins without affecting either Rev or Rex function, thus strongly suggesting a shared cofactor. This prediction was recently confirmed with the identification of the Rev/Rex activation Domain-Binding (Rab) protein (1), also termed the human Rev-interacting protein (hRIP) (13), a novel human nucleoporin-like factor that specifically binds the activation domains of HTLV-1 Rex and HIV-1 Rev as well as functionally equivalent domains found in other lentivirus Rev proteins. A novel nuclear pore-associated yeast protein has also been shown to bind the Rev and Rex activation domains specifically (37). Recently, the Rev activation domain (Fig. 1) has also been shown to function as an autonomous nuclear export signal (NES) that induces the specific export of proteins from the nuclei of both mammalian and amphibian cells when present in *cis* (11, 31, 40). Overall, these data suggest that Rev and Rex mediate RNA export from the mammalian cell nucleus by directing target RNAs to the nuclear pore via a specific interaction with Rab and, potentially, other nucleoporin-like cofactors (1, 13, 37).

Although the Rex activation domain has been previously localized to an ~21-amino-acid (aa) segment, located between residues 79 and 99 of the 189-aa Rex protein, by definition of the minimal sequence capable of replacing the Rev activation domain in *cis* (21, 39), very little is known about the sequence requirements for Rex activation domain function. Indeed, previous work has identified only one critical residue: leucine 90 (Fig. 1) (21). In addition, the Rex activation domain, while functionally equivalent to the Rev activation domain in terms of induction of viral structural protein expression, has not been shown to form an NES. To address these unresolved questions, we have used nuclear microinjection of proteins bearing wild-type or mutant forms of the Rex activation domain to confirm that there is indeed a direct correlation between Rex activation domain and NES function. Second, we have attempted to de-

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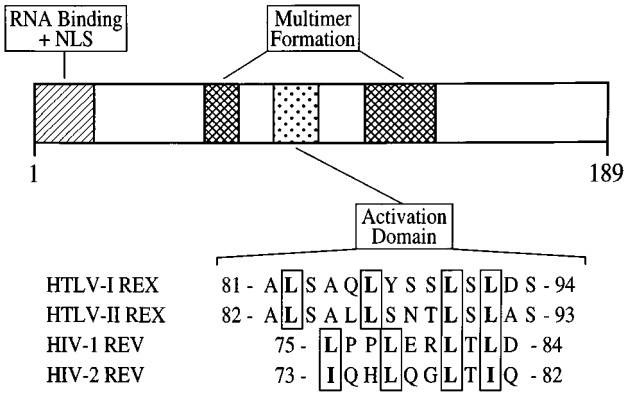


FIG. 1. Domain organization of HTLV-1 Rex. Sequences previously shown to be critical for different aspects of the biological activity of the 189-aa Rex protein are boxed. Also shown is the sequence of the minimal Rex NES defined in this work. Known or proposed functionally similar domains found in HTLV-2 Rex and HIV-1 and HIV-2 Rev are shown for comparison. The only residue in the Rex activation domain previously shown (21) to be critical for function is leucine 90, while leucines 78, 81, and 83 are each critical for HIV-1 Rev function (28). NLS, nuclear localization signal.

fine the potential sequence variation in the Rex activation domain/NES by using a novel randomization-selection screen predicated on the previous finding (1, 13) that the cellular Rab protein binds the Rex activation domain/NES specifically in vivo. If this finding is generally valid, then Rex clones containing random mutations in the activation domain that are selected on the basis of their ability to bind Rab should also retain Rex activation domain/NES function. Our data not only fully confirm this hypothesis but have also permitted the isolation of a set of highly diverse yet fully functional Rex activation domains/NESs. Interestingly, one of these selected sequences was very similar to the activation domain/NES found in HIV-1 Rev, while a second bore striking homology to the NES present in protein kinase inhibitor α (PKI), a cellular protein that plays no role in nuclear RNA export (40). On the basis of these results, we define a consensus Rex activation domain/Rab binding site and propose that this consensus sequence is also predictive of NES function.

## MATERIALS AND METHODS

**Mammalian cell microinjection.** Glutathione-*S*-transferase (GST) fusion protein expression plasmids based on pGEX-2T (Pharmacia) were constructed. Plasmid pGST:Rex encodes GST linked to residues 81 to 94 of wild-type Rex (Fig. 1), while the fusion protein encoded by pGST:L3+4A is identical except that leucines located at Rex residues 90 and 92 have been mutated to alanines. Plasmid pGST:2R encodes GST linked to the sequence $NH_2$-AFSRSFNAILH VSS-COOH, while pGST:2R/F82A encodes a fusion protein in which the second position of this sequence has been mutated from phenylalanine to alanine. In each case, GST was linked to the fused sequence via a hinge consisting of two glycine residues. All GST-containing proteins were purified by standard procedures in the absence of any detergents (31, 40). Two days prior to microinjection, HeLa cells were seeded at a density of $5 \times 10^5$ cells per 60-mm-diameter dish. GST fusion proteins were then injected at a final concentration of 3 mg/ml, and the cells were returned to 37°C. Each protein preparation was supplemented with 3 mg of rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch) per ml so that the site of injection could be determined. At 30 min after injection, the cells were fixed with 3% paraformaldehyde, and the subcellular localization of the injected proteins was determined by double-label indirect immunofluorescence. GST-containing proteins were detected by incubation with an anti-GST monoclonal antibody (Santa Cruz Biotechnology) followed by a goat antimouse antibody conjugated to rhodamine (Cappel); IgG was visualized with a donkey antirabbit antibody conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch). Samples were observed by indirect immunofluorescence at a magnification of ×400.

**Construction of randomized Rex libraries.** The GAL4-R4 randomized Rex sequence library was constructed by using a recombinant PCR technique. The Rex cDNA was amplified in two parallel reactions with primer pairs 5′-CCAT GGGAATTCTACATCGTCACGCCC-3′ (primer I)-5′-GAACTGTAGAGCT GAGCCGATA-3′ (primer II) and 5′-TATCGGCTCAGCTCTACAGTTCC $N_{12}$CCCCTCCTTCCCCACCC-3′ (primer III)-5′-GCATGCGTCGACTCAGT GGAATGTTGGGGG-3′ (primer IV), where $N_{12}$ refers to an equal mixture of all four deoxynucleotides at 12 base positions. The two overlapping primary amplification products obtained from these reactions were gel purified, and ~50-ng portions of each recovered fragment were combined and then reamplified with primers I and IV. Primer I introduces a unique *Eco*RI site into the Rex cDNA at codon 59 in Rex, while primer IV introduces a translation termination codon at codon 122 followed by a unique *Sal*I site (these sites are underlined in primer sequences). After cleavage of the resultant Rex PCR product with *Eco*RI and *Sal*I, the randomized Rex sequence (aa 59 to 121) was cloned into the yeast expression plasmid pGBT9 (Clontech) in frame with the encoded GAL4 DNA binding domain.

The GAL4-R8 randomized Rex library was generated by a single-step PCR procedure. The primers used were 5′-CCCCATCGATGGACGCGN$_{24}$TTATC CCTCGACTCC (primer V) and primer IV (described above). These primers allowed the generation of a PCR fragment encoding Rex aa 78 to 121 with positions 82 to 89 randomized. Primer V included a *Cla*I site, which occurs naturally in the Rex gene, while primer IV again introduced a *Sal*I site. These sites permitted the efficient cloning of these PCR products into the pGBT9-Rex(59-121) yeast expression plasmid in place of the wild-type Rex cDNA sequence.

The ligated GAL4-R4 and GAL4-R8 Rex libraries were phenol-chloroform extracted, ethanol precipitated, and then electroporated into XL-Blue bacteria (Stratagene). After selection for ampicillin-resistant transformants, each library was found to consist of $>5 \times 10^5$ independent clones. DNA derived from each pooled library was then introduced into the *Saccharomyces cerevisiae* indicator strain Y190 (18) along with the pVP16/Rab expression plasmid (1). Yeast transformants expressing Rex proteins that retained the ability to interact with Rab were then identified as previously described (1).

**Construction of mammalian Rex expression plasmids.** Plasmid pcRex, which expresses a full-length Rex cDNA sequence (aa 1 to 189) under the control of the human cytomegalovirus immediate-early promoter, has been described previously (36). Also previously described are plasmids pcRex/M12 and pcRex/M18, which express fully active mutant variants of the Rex protein containing missense mutations at aa 111 to 113 or aa 182 to 183, respectively, that each also introduce a unique *Bgl*II site (35).

Variant Rex genes derived from the R4 or R8 library screen were introduced into pcRex/M12 by PCR amplification of the Rex sequences present in the pGBT9-Rex(59-121) yeast expression plasmids, using primer I and a second primer that introduced a *Bgl*II site at the same site found in pcRex/M12. The resultant amplified Rex DNA sequences were then cleaved with *Cla*I and *Bgl*II and cloned into pcRex/M12 in place of the wild-type Rex sequence.

The various Rex mutants described in Tables 3 and 4 were generated by PCR amplification with a primer that traversed the Rex *Cla*I site and activation domain and a second primer that spanned the unique *Bgl*II site present in pcRex/M18. The first primer was also designed to introduce the desired mutation of the Rex activation domain. Amplified Rex fragments were cloned into pcRex/M18 after cleavage with *Cla*I and *Bgl*II.

Yeast expression plasmids containing the Rex mutations listed in Tables 3 and 4 were generated by PCR amplification of the Rex mutant sequences from the relevant mammalian expression plasmid by using primers I and IV (described above) followed by cloning into pGBT9.

All clones were sequenced by the dideoxy chain termination method with the Sequenase version 2.0 sequencing kit (United States Biochemical).

**Analysis of Rex activity and expression.** The abilities of Rex variants to bind Rab in the yeast two-hybrid assay context (9) were determined in the yeast indicator strain Y190 by analysis of induced β-galactosidase expression levels, as previously described (1, 18). All positive Rex clones (see Tables 1 to 4) displayed high levels of induced β-galactosidase activity, with little variation among positive clones, while all negative clones were indistinguishable from negative controls.

The abilities of Rex variants to function in mammalian cells were determined as previously described by using the pDM128/RXRE indicator construct (15, 21). Plasmid pDM128/CMV contains the chloramphenicol actyltransferase (CAT) indicator gene and the HIV-1 Rev response element located between two HIV-1-derived splice sites (22, 28). Cytoplasmic expression of the unspliced form of this RNA, and hence CAT expression, is dependent on Rev function. To construct pDM128/RXRE, the Rev response element was first deleted and then replaced by a polylinker sequence, to give the previously described pDM128/CMV/PL plasmid (12). The full-length RXRE was then amplified by PCR and inserted into the polylinker at unique *Asp*718 and *Xba*I sites. As previously reported (15), efficient CAT expression from the pDM128/RXRE indicator plasmid is dependent on Rex coexpression as assayed by cotransfection into the cell line COS.

COS cell cultures (35-mm-diameter dishes) were transfected with 25 ng of pDM128/RXRE, 600 ng of the relevant pcRex derivative, and 25 ng of the pBC12/CMV/β-gal internal control plasmid (1). CAT activities were determined at 48 h after transfection by the diffusion method (33). Western blot (immuno-

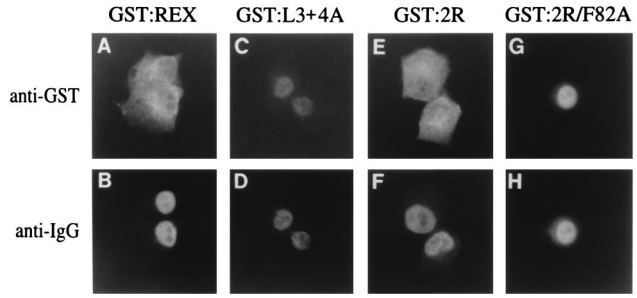


FIG. 2. The Rex activation domain is an NES. Purified recombinant fusion proteins consisting of GST linked to the wild-type 14-aa Rex activation domain (GST:REX), to the defective L3+4A activation domain mutant (GST:L3+4A), to the selected 2R activation domain (GST:2R), or to an inactive mutant of 2R (GST:2R/F82A) were microinjected into HeLa cell nuclei along with rabbit IgG. After incubation at 37°C for 30 min, the cells were fixed and subjected to double-label indirect immunofluorescence to visualize the intracellular locations of the two injected proteins.

blot) analysis of Rex protein expression was performed with a polyclonal rabbit anti-Rex antiserum (2).

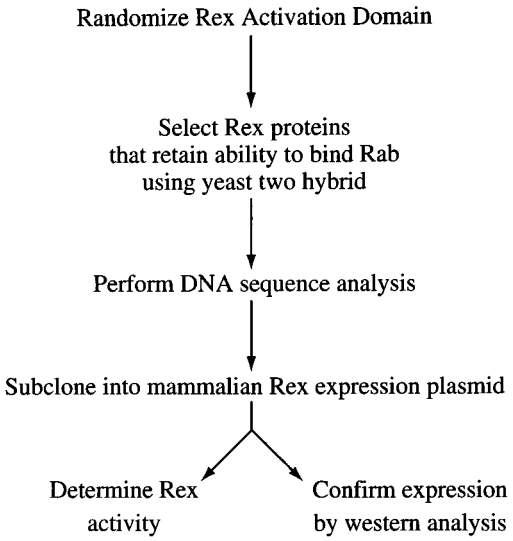


FIG. 3. Schematic outline of the yeast two-hybrid assay-based randomization-selection assay used here to analyze the HTLV-1 Rex activation domain. See text for a detailed discussion.

## RESULTS

**The Rex activation domain is an NES.** To test whether the Rex activation domain forms an NES, we used a previously described assay (31, 40) in which wild-type or mutant sequences to be tested for NES activity are first fused to the carboxy terminus of GST, expressed in bacteria, and then purified by affinity chromatography. The GST:Rex fusion protein contains GST fused to the 14-aa Rex activation domain sequence shown in Fig. 1. The GST:L3+4A fusion protein is identical to GST:Rex except that leucines located at Rex residues 90 and 92 have been mutated to alanines. This mutation blocks Rex activation domain function (see below). These recombinant proteins were each mixed with rabbit IgG and then microinjected into the nuclei of HeLa cells. After 30 min of incubation at 37°C, the injected culture was fixed and subjected to double-label immunofluorescence to localize both the fusion protein and the IgG in the injected cells.

Both IgG and GST lack a nuclear localization signal and an NES, and both therefore normally remain within the cellular compartment into which they are microinjected (31, 40). The IgG served here as an internal control to verify that microinjected proteins were indeed introduced into the nucleus exclusively (Fig. 2B and D). If the sequence fused to GST lacks NES function, then this fusion protein will also remain nuclear, as seen with the GST:L3+4A fusion protein containing a nonfunctional activation domain mutant (Fig. 2C). In contrast, if the attached sequence is a functional NES, then the bulk of the GST fusion protein will be exported to the cytoplasm within the 30-min incubation period (31, 40). This is indeed seen with the wild-type GST:Rex fusion, which has become predominantly cytoplasmic (Fig. 2A) even though the coinjected IgG control remains exclusively nuclear (Fig. 2B). Therefore, it is apparent that the Rex activation domain is indeed similar to the Rev activation domain in forming an NES.

**Analysis of the Rex NES with a novel randomization-selection assay.** The Rex activation domain forms both an NES (Fig. 2) and a specific binding site for the cellular Rab cofactor (1, 13). If Rex activation domain function is dependent on both of these properties, then it should be possible to define the sequence requirements for both activation domain and NES function by using the in vivo randomization-selection assay outlined in Fig. 3. Small segments (12 to 24 bp) of the *rex* gene, which encode parts of the minimal Rex NES (Fig. 1 and 2), were randomized by PCR (see Materials and Methods for details) in a segment of Rex extending from aa 59 to 121. The resultant library was then expressed in the yeast indicator strain Y190 (18) as a fusion with the GAL4 DNA binding domain, as previously described (1). Yeast transformants, which were also engineered to express a previously described fusion protein consisting of the VP16 transcription activation domain linked to the full-length human Rab protein (1), were then selected for expression of the *his3* indicator gene (18). Yeast colonies able to grow on the selection plates were picked, and Gal4-Rex library plasmids were isolated. These were then reintroduced into the yeast Y190 indicator cells in either the presence or the absence of the VP16-Rab expression plasmid. These secondary transformants were then analyzed for the level of expression of a second indicator gene, *lacZ*, to confirm that the randomized Rex sequence was indeed able to bind Rab specifically. Positive clones were subjected to DNA sequence analysis prior to being subcloned into the mammalian Rex expression plasmid pcRex (36) and analyzed for Rex function.

**Ability to bind Rab predicts Rex activation domain function.** The first randomization analysis focused on Rex aa 90 to 93, which includes the known critical leucine at position 90. Approximately 50,000 transformants were screened, resulting in the identification of the 24 independent Rex clones listed in Table 1. In addition, we also randomly selected four Rex clones that did not show any ability to bind Rab, as well as a Rex mutant with a mutation in this region, Rex ΔAD, that was previously shown to lack Rex function and not to bind Rab (1, 21).

The 24 Rex clones obtained encoded 18 different sequences across this 4-aa segment of the Rex activation domain. Redundant clones were not truly identical, in that all but one displayed a different underlying nucleotide sequence. In addition, the apparently wild-type clone R4-14 is also novel, in that it differs from the wild type at the nucleotide level (Table 1). Importantly, all of the selected clones were, when tested, highly active as Rex proteins, while the nonselected Rex mutants R4-19 to R4-22 were inactive. This did not reflect differ-

TABLE 1. Activities of R4 series Rex variants[a]

| Clone tested | Randomized sequence (residues 90 to 93) | Rab binding | Rex activity[b] |
|---|---|---|---|
| Rex | L S L D | + | 100 |
| R4-1 | L W I T | + | 60 ± 15 |
| R4-2 (×2) | L V I G | + | 81 ± 14 |
| R4-3 | L W V S | + | 65 ± 20 |
| R4-4 | L T L Q | + | 105 ± 17 |
| R4-5 | L K L E | + | 111 ± 23 |
| R4-6 | L S I S | + | 87 ± 13 |
| R4-7 | L H V S | + | 84 ± 17 |
| R4-8 (×2) | L V L S | + | 79 ± 28 |
| R4-9 (×2) | L V L G | + | 61 ± 18 |
| R4-10 | L H I G | + | 65 ± 24 |
| R4-11 (×2) | L R L S | + | 53 ± 22 |
| R4-12 | L W I G | + | 80 ± 24 |
| R4-13 | L V I T | + | 62 ± 15 |
| R4-14 | L S L D | + | ND[c] |
| R4-15 | L Y V S | + | ND |
| R4-16 | V W V S | + | 38 ± 8 |
| R4-17 (×2) | C T I S | + | 87 ± 24 |
| R4-18 (×2) | C K I * | + | ND |
| R4-19 | V T G G | − | 1.4 ± 1.2 |
| R4-20 | G G K T | − | 4.9 ± 4.3 |
| R4-21 | Q V Y G | − | 1.3 ± 1.3 |
| R4-22 | A E V G | − | 0.4 ± 0.6 |
| ΔAD | G G G G | − | 1.8 ± 1.4 |

[a] Randomization of residues 90 to 93 of Rex, followed by selection for retention of Rab binding, resulted in the identification of 24 Rex variants displaying the 18 protein sequences shown (R4-1 to R4-18). Six clones were obtained in duplicate, as indicated by ×2, while one clone contained a translation termination codon, indicated by an asterisk. Four Rex variants unable to bind Rab (R4-19 to R4-22) were picked at random, while the ΔAD Rex mutant was included as a negative control.

[b] Activities are given relative to that of Rex, which was arbitrarily set at 100, and represent averages from three independent experiments ± the observed standard deviation. All transfections were internally controlled and showed little experimental variation.

[c] ND, not determined.

ential stability, as the four inactive Rex mutants were expressed at levels comparable to those of the active Rex mutants, as determined by Western analysis (data not shown). Clone R4-18 was not examined, as the termination codon at position 93 would result in the loss of carboxy-terminal Rex sequences that are known to be critical for other aspects of Rex function (Fig. 1). Similarly, R4-15 was also omitted because of a large deletion mutation located immediately 3′ to the Rex activation domain. Finally, R4-14 was not tested because it is effectively wild-type Rex.

Inspection of the sequences obtained by this randomization-selection protocol confirmed that leucine 90 is indeed important, in that it was retained in 19 of the 24 clones. All six possible leucine codons were utilized. A single clone was found with valine at position 90. However, this conservative substitution resulted in a Rex clone displaying the lowest activity observed in this series (Table 1).

The isolates R4-17 and R4-18, each obtained twice, were unexpected in that they feature a cysteine at position 90. R4-17 was found to encode an essentially fully active Rex protein. Analysis of amino acid substitution patterns for leucine across species boundaries have suggested Ile = Val > Met > Phe as appropriate replacements, with cysteine not favored (7). Nevertheless, cysteine is clearly able to substitute for leucine in this case, a finding that is no doubt also dependent on amino acid context. Interestingly, isoleucine was not detected at position 90 in this analysis, as also predicted by earlier work showing a significant loss of function in such a Rex missense mutant (21).

By analogy to Rev, one would predict that leucine 92 would also be important for Rex function (28). Indeed, only very conservative substitutions are detected at this position, with six leucines, eight isoleucines, and four valines observed in the 18 distinct sequences obtained (Table 1). Given that leucine is encoded by twice as many codons as isoleucine, this finding implies that isoleucine is fully as appropriate as leucine at this position for maintenance of Rex activation domain function. In fact, isoleucine is observed at this relative position in the HIV-2 Rev activation domain (Fig. 1) and in the PKI NES (see below).

The sequence variability observed at position 91 in these selected Rex clones is less easy to interpret. However, the prevalence of the uncommon amino acid tryptophan (4 of 18) is striking. It is also notable that certain common amino acids, such as glycine, leucine, and proline, were not detected. However, the significance of this exclusion is not clear, given the relatively small size of this sample.

The last randomized position analyzed, i.e., position 93, shows a high prevalence of small or hydrophilic amino acids, such as serine and glycine, and, in one case, a stop codon. This latter result strongly suggests that the last critical residue for Rex activation domain function is leucine 92, a result also reported previously (10, 28) for the equivalent leucine 83 in the HIV-1 Rev activation domain (Fig. 1). The small sizes of the residues selected at position 93 in this screen may therefore largely reflect a requirement that this residue not sterically obstruct critical protein-protein interactions involving large hydrophobic amino acids located immediately amino terminal.

The data presented in Table 1 suggest that two positions within this 4-aa segment are selected for, i.e., leucine at 90 and leucine or isoleucine at 92, while the selection is largely against deleterious residues at positions 91 and 93. Consistent with this hypothesis, we observed 24 positive clones of 50,000, i.e., ~1 of 2,000 total clones or ~1 of 1,800 of these clones retaining an open reading frame through Rex residue 92. In contrast, a simple selection for leucine at position 90 and leucine or isoleucine at 92 predicts a success rate of ~1 in 80. It is therefore clear that many residues, or combinations of residues, at positions 91 and 93 are inconsistent with efficient Rab binding.

The second selection focused on randomization of residues 82 to 89 of Rex. We screened ~100,000 yeast transformants and obtained 11 positive transformants. Two negative clones were also chosen randomly as controls. As shown in Table 2, we again found that all Rex proteins that retained the ability to bind Rab also retained substantial Rex function, while the two nonselected controls lacked both Rex function and Rab binding ability.

Inspection of the 11 functional Rex sequences obtained in this analysis showed a considerable amount of sequence variation, with no invariant amino acid at any position studied. However, leucine was highly prevalent at position 83, while leucine and the four similar large hydrophobic amino acids isoleucine, valine, methionine, and phenylalanine were favored at residue 87. It therefore appeared that the two critical large hydrophobic residues observed at positions 82 and 86 in wild-type Rex had effectively been moved to positions 83 and 87 in the selected clones, thus changing the "3-3-1" leucine spacing seen in Rex to a novel 3-2-1 spacing. Exceptions to this generalization included R8-9, which does not have a hydrophobic residue at position 83; R8-10, which has phenylalanine residues at positions 82 and 86; and R8-11, which has a leucine at position 87 but no large hydrophobic residue at either position 82 or 83, although a cysteine is observed at position 84. The

TABLE 2. Activities of R8 series variants[a]

| Clone tested | Randomized sequence (residues 82 to 89) | Rab binding | Rex function[b] |
|---|---|---|---|
| Rex | L S A Q L Y S S | + | 100 |
| R8-1 | S L I E G V R N | + | 127 ± 9 |
| R8-2 | T L S Q R F R T | + | 104 ± 28 |
| R8-3 | K L A L S L Q S | + | 88 ± 18 |
| R8-4 | S L E E G M M S | + | 34 ± 17 |
| R8-5 | V L S Q R M S C | + | 80 ± 28 |
| R8-6 | M L S D S L M D | + | 78 ± 10 |
| R8-7 | D L V S G I Q F | + | 50 ± 11 |
| R8-8 | V L S S Q F G Q | + | 53 ± 9 |
| R8-9 | L T C R L L M D | + | 67 ± 10 |
| R8-10 | F S R S F N A I | + | 65 ± 24 |
| R8-11 | E A C A A L E R | + | 34 ± 17 |
| R8-12 | C P G G P V C S | − | 1.0 ± 1.1 |
| R8-13 | E S R C V R L S | − | 2.9 ± 2.5 |

[a] Randomization of residues 82 to 89 of Rex, followed by selection for retention of Rab binding, led to the identification of the 11 Rex sequence variants shown (R8-1 to R8-11). Two additional randomized clones lacking the ability to bind Rab (R8-12 and R8-13) were chosen at random. Leucine residues, as well as the similar large hydrophobic residues isoleucine, valine, methionine, and phenylalanine, are boxed to emphasize clustering at residues 83 and 87.

[b] See Table 1, footnote *b*.

TABLE 3. Activities of selected Rex mutants[a]

| Clone tested | Protein sequence (residues 82 to 93) | Rex function[b] | Rab binding |
|---|---|---|---|
| Rex | L S A Q L Y S S L S L D | 100 | + |
| Q85P | – – – P – – – – – – – – | <5 | − |
| Q85A | – – – A – – – – – – – – | 82 | + |
| S91P | – – – – – – – – – P – – | <5 | − |
| L2+3A | – – – – A – – – A – – – | <5 | − |
| L3+4A | – – – – – – – – A – A – | <5 | − |
| 2R | F S R S F N A I L H V S | 97 | + |
| 2R/F82A | A – – – – – – – – – – – | <5 | − |
| 2R/S83A | – A – – – – – – – – – – | 74 | + |
| LS1 | S L S S S L S S L S L S | 74 | + |
| LS2 | – – – – L S – – – – – – | <5 | − |

[a] The indicated Rex activation domain mutants were tested for biological activity in COS cells and for Rab binding ability in *S. cerevisiae*.

[b] Activity relative to that of Rex.

R8-11 isolate is particularly interesting in that it contains a sequence (LERLSLD; positions 87 to 93) that is essentially identical to the core activation domain of HIV-1 Rev (LERLTLD; positions 78 to 84) (Fig. 1).

While the Rex activation domain sequences listed in Table 2 are intriguing, they are too limited to allow statistically significant conclusions about sequence preferences to be made. Nevertheless, the low number of positive clones obtained in this screen (11 of 100,000; i.e., ~1 of 9,000 total or ~1 of 6,130 of clones retaining a Rex open reading frame) clearly indicates significant sequence constraints in addition to, for example, a selection for leucine at position 83 and for a large hydrophobic amino acid at position 87. In this context, it is of interest that proline was rigorously excluded from all positive clones in both screens.

**Analysis of selected Rex activation domain mutants.** We next constructed and analyzed a small number of Rex mutants suggested by the data presented in Tables 1 and 2. Initially, we substituted single proline residues into the Rex activation domain at position 85 (Q85P) or position 91 (S91P). These two mutations, which do not directly affect any of the apparently critical leucine residues, were found to entirely block both Rab binding and Rex function (Table 3). In contrast, a control alanine substitution at position 85 (Q85A) was phenotypically silent. This result, while predicted by the exclusion of proline from the functional Rex activation domain clones listed in Tables 1 and 2, is nevertheless somewhat surprising given that the functionally equivalent Rev activation domain contains two proline residues (Fig. 1). On the other hand, and as predicted from both earlier work on HIV-1 Rev (28) and the data presented in Tables 1 and 2, substitution of alanine for leucine residues 86 and 90 (L2+3A) or residues 90 and 92 (L3+4A) blocked both Rab binding and Rex function. In all cases, these mutations had no affect on Rex protein stability in vivo (Fig. 4).

To address whether these 4- and 8-aa randomized sequences could be combined with one another and still retain function, we next constructed a Rex protein, termed 2R, containing the 8-aa randomized sequence R8-10 and the 4-aa sequence R4-7. Although this combined sequence retains only a single leucine residue and is identical to wild-type Rex at only two positions (Ser-83 and Leu-90), it nevertheless functioned effectively as a Rex activation domain and also bound Rab efficiently (Table 3). However, replacement of Phe-82 by alanine (2R/F82A) abrogated both of these activities, thus supporting the hypothesis that this phenylalanine residue is functionally substituting for the leucine residue normally found at this location in wild-type Rex. In contrast, mutation of Ser-83 (one of two residues shared with wild-type Rex) to alanine (2R/S83A) only modestly affected Rex function and Rab binding (Table 3). These mutations again had no effect on Rex protein stability (Fig. 4).

As noted above, the randomization data presented in Tables 1 and 2 are consistent with the hypothesis that efficient Rab binding requires not only leucines or other large hydrophobic residues at four critically spaced positions but also selection against certain deleterious residues, such as proline, at the intervening positions. If this hypothesis is valid, it should then be possible to design a very simple NES consisting only of leucine and a second amino acid that is compatible with NES function.

To test this hypothesis, we constructed two artificial NES sequences consisting of only leucine and serine. The first of these, termed LS1, has the 3-2-1 spacing of the four leucine residues that appeared to be favored in the selection described for Table 2. The second, termed LS2, is identical to LS1 except that the spacing of the leucine residues was changed to 2-3-1. As shown in Table 3, the LS1 sequence was indeed functional as a Rex activation domain and also mediated efficient Rab binding. In contrast, the similar LS2 sequence, which differs only in the order of two core residues, was neither functional as an activation domain nor able to bind Rab. These data there-

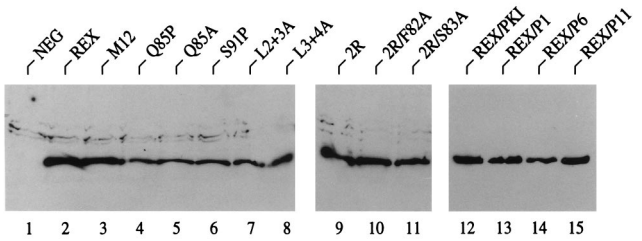


FIG. 4. Western analysis of the levels of expression of the indicated Rex variants and Rex/PKI chimeras in transfected COS cell cultures. NEG, negative control.

TABLE 4. Activities of Rex/PKI chimeras[a]

| Clone tested | Protein sequence | Rex function[b] | Rab binding |
|---|---|---|---|
| Rex | L S A Q L Y S S L S L D | 100 | + |
| R8-3 | K L A L S L Q S L S L D | 89 | + |
| PKI | E L A L K L A G L D I N | 104 | + |
| PKI/P1 | A – – – A – – – – – – – | 154 | + |
| PKI/P6 | – A – – – A – – – – – – | <5 | – |
| PKI/P11 | – – – – – – – – A – A – | <5 | – |

[a] The indicated wild-type and mutant PKI NES sequences (residues 36 to 47) were introduced into Rex in place of the wild-type Rex sequence from residue 82 to 93, and the resultant chimeras were tested for Rex function in primate cells and for Rab binding in *S. cerevisiae.*

[b] Activity relative to that of Rex.

fore emphasize the critical importance of the spacing of the large hydrophobic residues within NES sequences and also show that a functional NES can indeed be constructed with only leucine and a second compatible amino acid, such as serine.

**Rab binding predicts NES function.** As noted above, the fully functional 2R activation domain sequence selected on the basis of Rab binding retains only a single leucine residue and has very little overall sequence identity with the wild-type Rex sequence (Table 3). To address whether the 2R sequence also forms a functional NES, we fused the 2R sequence to the carboxy terminus of GST and microinjected this GST:2R fusion protein into HeLa cell nuclei, after first adding rabbit IgG as an internal control. As shown in Fig. 2, the GST:2R fusion protein was indeed predominantly cytoplasmic after a brief, 30-min incubation at 37°C (Fig. 2E), while the coinjected IgG remained entirely nuclear (Fig. 2F). In contrast, a similar GST fusion protein bearing the defective 2R/F82A activation sequence, in which the first phenylalanine residue of the 2R sequence was changed to alanine (Table 3), remained entirely nuclear (Fig. 2G). Therefore, it is apparent that the 2R sequence, which contains only one leucine residue, is not only a functional Rex activation domain but also an active NES. Similarly, the LS1 activation domain variant described in Table 3 was also observed to function as an NES when assayed by microinjection (data not shown).

**A cellular NES can functionally replace the Rex activation domain.** A striking result shown in Table 2 is that, in the selected randomized clones, the spacing of the critical leucine (or large hydrophobic) residues had generally changed from the 3-3-1 spacing seen in wild-type Rex to a novel 3-2-1 spacing. This is highly reminiscent of the spacing of the four critical leucine and isoleucine residues observed in the NES of the human PKI protein (Table 4) (40). Indeed, one of the selected randomized clones, R8-3, was found to have extensive sequence homology with the PKI NES, thus suggesting that the Rex activation domain and the PKI NES might be functionally equivalent.

To test this hypothesis, we replaced the 12-aa Rex activation domain sequence shown in Table 4 with the full-length, 12-aa PKI NES sequence. This chimeric Rex/PKI protein was found to be fully active as a Rex protein and also to bind Rab efficiently in *S. cerevisiae*. A previously described (40) PKI mutant, termed P1 (Table 4), that retains NES function also retained Rex function and Rab binding. In contrast, two mutations, termed P6 and P11, that inactivate the PKI NES by changing critical NES leucine or isoleucine residues to alanine (40) also lacked both detectable Rex activity and Rab binding ability (Table 4). All Rex/PKI chimeras were, however, found to be equivalently stable in transfected cells (Fig. 4). Therefore, the NES found in the cellular protein PKI is fully able to substitute for the Rex activation domain in mediating both the nuclear export of target RNA species and binding to the cellular Rab cofactor.

## DISCUSSION

The observation that the Rex activation domain forms an NES that will induce the nuclear export of proteins when present in *cis* (Fig. 2) was not unexpected given the finding that the functionally equivalent activation domain of HIV-1 Rev is an NES (10, 40). Both the HTLV-1 Rex and the HIV-1 Rev activation domains also form binding sites for the human Rab protein (1, 13), a property shared by the functionally equivalent activation domains (1, 12, 28, 30) found in visna-maedi virus Rev and equine infectious anemia virus Rev, sequences that have recently also been shown to function as NESs (31). Therefore, it is apparent that the activation domains found in the Rev/Rex class of retroviral posttranscriptional regulatory proteins share three properties; i.e., they are functionally interchangeable, they form NESs, and they bind the cellular Rab cofactor.

Given that these three properties are indeed closely tied, we then hypothesized that it should be possible to define the permissible sequence variation in a functional activation domain/NES by using the yeast two-hybrid assay to identify clones within a randomized Rex NES sequence library that retained the ability to bind Rab (Fig. 3). This prediction indeed proved to be accurate, in that all mutants of the Rex activation domain that were selected on the basis of their ability to bind Rab also retained activation domain function, while randomly selected mutants that did not bind Rab lacked Rex function (Tables 1 and 2). Although we have not confirmed that all functional variants of the Rex activation domain identified in this work also retain NES function, we did test one highly variant Rex activation domain sequence, termed 2R, that retains only one leucine residue of the four observed in the wild-type Rex sequence yet is still able to bind Rab and promote Rex function (Table 3). As shown in Fig. 2, this variant sequence forms an effective NES, while a point mutation of the 2R sequence, termed 2R/F82A, that blocks Rab binding and Rex function also eliminates NES activity (Table 3 and Fig. 2). We believe it is therefore evident that all of the functional Rex activation domains that were selected on the basis of Rab binding are also functional NES sequences.

If functional activation domains form NES sequences, does this imply that an NES sequence from a cellular protein can serve as a Rex activation domain? At least for the NES of the cellular protein PKI (40), this is clearly true (Table 4). In particular, it is apparent that functional forms of the PKI NES can fully substitute for the Rex activation domain, while mutant forms of the PKI sequence that lack NES function can not. In addition, it is of interest that the PKI NES also binds to the Rab protein (Table 4), thus extending the correlation of NES function and Rab binding to at least one cellular protein.

The data reported in this paper demonstrate that the ability to bind Rab is predictive of NES function and therefore strongly support the hypothesis that Rab mediates the NES activity observed for the activation domains present in the Rev/Rex class of nuclear RNA export factors. However, these data do not predict that all functional NES sequences will bind Rab. For example, an NES sequence might act along one of the other nuclear export pathways that have been proposed to exist in eukaryotic cells (10, 24) and might therefore utilize different cofactors. Indeed, the NES recently identified in the

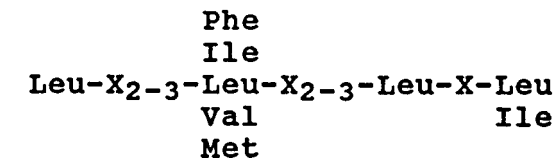


FIG. 5. Consensus sequence for a functional Rev/Rex activation domain/NES. This sequence was derived by inspection of the various Rex activation domain variants and mutants described in this paper, as well as of functional activation domains found in nature. However, some activation domains/NESs, such as the one observed in equine infectious anemia virus Rev (12, 30, 31), clearly do not conform to this consensus.

heterogeneous nuclear ribonucleoprotein A1 (32) may fulfill this prediction in that it is very different in sequence from the Rev/Rex NESs, is unable to substitute for the Rev NES in mediating Rev function, and does not bind to Rab in the yeast two-hybrid setting (data not shown).

The sequences shown in Tables 1 and 2 were selected on the basis of their ability to mediate a readily detectable level of Rab binding. Sequences that bind Rab more weakly might not have been detected with this assay protocol yet might nevertheless retain substantial NES activity. It is therefore likely that the number of positive clones detected in this assay is an underestimate of the total number of functional Rex activation domains/NESs that were present in the two randomized Rex libraries. Nevertheless, it is also true that randomized Rex sequences that retained the ability to bind Rab tightly were rare. On the basis of the frequency of positive clones observed for the two sequence libraries analyzed here, we would predict that if we were to simultaneously randomize the entire 12-aa Rex activation domain sequence analyzed in this study, then among clones that retained an open Rex reading frame, only $1/1{,}800 \times 1/6{,}130$, i.e., less than 1 in $10^7$ clones, would express a Rex protein that bound Rab efficiently and was therefore likely to retain a fully functional activation domain/NES. These observations imply that sequences able to efficiently substitute for the Rex activation domain are very unlikely to be encountered by chance. The detection of such a functionally equivalent sequence in another viral or cellular protein would therefore suggest that this protein contains an NES of the Rev/Rex class that plays a role in the normal function of that protein in vivo.

**Sequence requirements for Rex activation domain/NES function.** Inspection of the amino acid sequences observed in functional Rex and Rev activation domains/NESs, including the novel sequences reported in this paper, clearly indicates that such NESs share certain characteristics, of which the most obvious is the presence of four critically spaced leucines or other large hydrophobic amino acids. These data also suggest that the spacings 3-3-1 seen in Rex, 3-2-1 seen in PKI, and 2-2-1 seen in Rev are acceptable, while other spacings, including 2-3-1 (Table 3), are not. It is also apparent that there is no tight consensus sequence for NES function. In this respect, NESs appear to be similar to nuclear localization signals, which consist of several basic residues within a short motif that cannot be readily expressed as a consensus sequence (4). It is nevertheless possible to suggest a loose NES consensus sequence, as shown in Fig. 5. In this sequence, the letter X is intended to imply a lack of strong selection for a particular amino acid at the indicated positions and not that any amino acid is suitable, as this is clearly not the case (Table 3). However, X can be a single amino acid, such as serine. While this consensus sequence would include the majority of the functional activation domains/NESs reported in this paper or identified by earlier work (21, 28, 39, 40), it does not accommodate all known functional Rev NES sequences. In particular, it does not include the unusual activation domain/NES observed in equine infectious anemia virus Rev (12, 30, 31), which shows no homology to the leucine-rich NESs shown in Fig. 1. Nevertheless, we would suggest that this consensus sequence does provide a useful guide when analyzing proteins of interest for potential NES sequences. Using this approach, we have identified sequences within several cellular proteins, in addition to the PKI NES, that can both functionally substitute for the Rev or Rex activation domain and also act as an NES (data not shown). It therefore appears probable that NESs that conform to the loose consensus shown in Fig. 5 will eventually be identified on many cellular proteins that move between the eukaryotic nucleus and cytoplasm.

## ACKNOWLEDGMENTS

We thank Michael Malim for helpful discussions and for communication of data prior to publication.

This work was funded by the Howard Hughes Medical Institute.

## REFERENCES

1. **Bogerd, H. P., R. A. Fridell, S. Madore, and B. R. Cullen.** 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. Cell **82:**485–494.
2. **Bogerd, H. P., and W. C. Greene.** 1993. Dominant negative mutants of human T-cell leukemia virus type I Rex and human immunodeficiency virus type 1 Rev fail to multimerize in vivo. J. Virol. **67:**2496–2502.
3. **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 the type 1 human immunodeficiency virus Rev RNA response elements. Proc. Natl. Acad. Sci. USA **88:**5704–5708.
4. **Boulikas, T.** 1993. Nuclear localization signals (NLS). Crit. Rev. Eukaryot. Gene Expression **3:**193–227.
5. **Cullen, B. R.** 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. Microbiol. Rev. **56:**375–394.
6. **Daly, T. J., K. S. Cook, G. S. Gray, T. E. Maione, and J. R. Rusche.** 1989. Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element *in vitro*. Nature (London) **342:**816–819.
7. **Dayhoff, M. O., R. V. Eck, and C. M. Park.** 1972. A model of evolutionary change in proteins, p. 89–99. *In* Atlas of protein sequence and structure. National Biomedical Research Foundation, Silver Spring, Md.
8. **Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. Proc. Natl. Acad. Sci. USA **86:**1495–1499.
9. **Fields, S., and O.-K. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
10. **Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Lührmann.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:**475–483.
11. **Fischer, U., S. Meyer, M. Teufel, C. Heckel, R. Lührmann, and G. Rautmann.** 1994. Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. EMBO J. **13:**4105–4112.
12. **Fridell, R. A., K. M. Partin, S. Carpenter, and B. R. Cullen.** 1993. Identification of the activation domain of equine infectious anemia virus Rev. J. Virol. **67:**7317–7323.
13. **Fritz, C. C., M. L. Zapp, and M. R. Green.** 1995. A human nucleoporin-like protein that specifically interacts with HIV Rev. Nature (London) **376:**530–533.
14. **Grassmann, R., 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.
15. **Gröne, M., E. Hoffmann, S. Berchtold, B. R. Cullen, and R. Grassmann.** 1994. A single stem-loop structure within the HTLV-I Rex response element is sufficient to mediate Rex activity *in vivo*. Virology **204:**144–152.
16. **Hammarskjöld, M.-L., J. Heimer, B. Hammarskjöld, I. Sangwan, L. Albert, and D. Rekosh.** 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. J. Virol. **63:**1959–1966.
17. **Hanly, S. M., L. T. Rimsky, M. H. Malim, J. H. Kim, J. Hauber, M. Duc Dodon, 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.
18. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993.

The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.

19. **Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. M. Green, J. Karn, A. D. Lowe, 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.
20. **Hidaka, M., J. Inoue, M. Yoshida, and M. Seiki.** 1988. Posttranscriptional regulator (Rex) of HTLV-I initiates expression of viral structural proteins but suppresses expression of regulatory proteins. EMBO J. **7:**519–523.
21. **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.
22. **Hope, T. J., X. Huang, D. McDonald, and T. G. Parslow.** 1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. Proc. Natl. Acad. Sci. USA **87:**7787–7791.
23. **Inoue, J.-I., M. Yoshida, and M. Seiki.** 1987. Transcriptional ($p40^{x}$) and posttranscriptional ($p27^{x\text{-}III}$) 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.
24. **Jarmolowski, A., W. C. Boelens, E. Izaurralde, and I. W. Mattaj.** 1994. Nuclear export of different classes of RNA is mediated by specific factors. J. Cell Biol. **124:**627–635.
25. **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.
26. **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.
27. **Malim, M. H., J. Hauber, S.-Y. Le, 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.
28. **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.
29. **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.
30. **Mancuso, V. A., T. J. Hope, L. Zhu, D. Derse, T. Phillips, and T. G. Parslow.** 1994. Posttranscriptional effector domains in the Rev proteins of feline immunodeficiency virus and equine infectious anemia virus. J. Virol. **68:** 1998–2001.
31. **Meyer, B. E., J. L. Meinkoth, and M. H. Malim.** 1996. Nuclear transport of the human immunodeficiency virus type 1, visna virus, and equine infectious anemia virus Rev proteins: identification of a family of transferable nuclear export signals. J. Virol. **70:**2350–2359.
32. **Michael, W. M., M. Choi, and G. Dreyfuss.** 1995. A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell **83:**415–422.
33. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–447.
34. **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.
35. **Rimsky, L., M. Duc Dodon, E. P. Dixon, and W. C. Greene.** 1989. *trans*-dominant inactivation of HTLV-I and HIV-1 gene expression by mutation of the HTLV-1 Rex transactivator. Nature (London) **341:**453–456.
36. **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:**738–740.
37. **Stutz, F., M. Neville, and M. Rosbash.** 1995. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. Cell **82:**495–506.
38. **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.
39. **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.
40. **Wen, W., J. L. Meinkoth, R. Y. Tsien, and S. S. Taylor.** 1995. Identification of a signal for rapid export of proteins from the nucleus. Cell **82:**463–473.
41. **Zapp, M. L., and M. R. Green.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature (London) **342:**714–716.