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Characterization of the Nuclear Export Signal of Human T-Cell Lymphotropic Virus Type 1 Rex Reveals that Nuclear Export Is Mediated by Position-Variable Hydrophobic Interactions

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We previously determined that amino acids 64 to 120 of human T-cell lymphotropic virus type 1 (HTLV-1) Rex can restore the function of an effector domain mutant of human immunodeficiency virus type 1 (HIV-1) Rev (T. J. Hope, B. L. Bond, D. McDonald, N. P. Klein, and T. G. Parslow, J. Virol. 65:6001–6007, 1991). In this report, we (i) identify and characterize a position-independent 17-amino-acid region of HTLV-1 Rex that fully complements HIV-1 Rev effector domain mutants and (ii) show that this 17-amino-acid region and specific hydrophobic substitutions can serve as nuclear export signals. Mutagenesis studies revealed that four leucines within the minimal region were essential for function. Alignment of the minimal Rex region with the HIV-1 Rev effector domain suggested that the position of some of the conserved leucines is flexible. We found two of the leucines could each occupy one of two positions within the context of the full-length HTLV-1 Rex protein and maintain function. The idea of flexibility within the Rex effector domain was confirmed and extended by identifying functional substitutions by screening a library of effector domain mutants in which the two regions of flexibility were randomized. Secondly, the functional roles of the minimal Rex effector domain and hydrophobic substitutions were independently confirmed by demonstrating that these effector domains could serve as nuclear export signals when conjugated with bovine serum albumin. Nuclear export of the wild-type Rex conjugates was temperature dependent and sensitive to wheat germ agglutinin and was blocked by a 20-fold excess of unlabeled conjugates. Together, these studies reveal that position-variable hydrophobic interactions within the HTLV-1 Rex effector domain mediate nuclear export function.

The Rex protein of human T-cell lymphotropic virus type 1 (HTLV-1) mediates the cytoplasmic localization of incompletely spliced and unspliced viral RNAs (24). Rex is a member of a family of functionally related proteins, generally known as the Rev-like proteins, which are found in complex retroviruses. The Rev-like proteins have at least two essential domains with unique functions, (i) a specific RNA binding activity that interacts with structural response elements within the viral mRNAs and (ii) an effector or activation domain that facilitates interaction with endogenous pathways (12, 17, 20). The effector domain of human immunodeficiency virus type 1 (HIV-1) Rev has recently been demonstrated to be a nuclear export signal (NES) (9, 31). A NES in the cyclic AMP (cAMP)-dependent protein kinase inhibitor has a sequence similar to those of the effector domains of Rev-like proteins (31). Studies of chimeric proteins have demonstrated that the effector domains of many Rev-like proteins can act in a heterologous context. For example, the effector domains of viral Rev-like proteins can complement the function of an effector domain mutant of HIV-1 or visna virus Rev (10, 12, 19, 29).

The identified effector domains of Rev-like proteins fall into two classes, as shown in Fig. 1. First, there are the typical effector domains found in HTLV-1, HIV, simian immunodeficiency virus (SIV), and visna virus. These domains are characterized by a 4-amino-acid region known as the core tetramer. This region is composed of the sequence LXLX, in which the last amino acid often has a negative charge (12). Positions −3 and −6 relative to the core tetramer can also be functionally occupied by methionine in visna virus (29) or isoleucine in HIV-2 and SIV (1) (Fig. 1). Adjacent leucines located in the region upstream of the core tetramer have been shown to be important in HIV-1 Rev (18). These upstream positions at −3 and −6 relative to the core tetramer are also conserved in HIV, SIV, and visna virus but not in HTLV-1 Rex.

Atypical effector domains are found in feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). These regions, like the typical effectors, have been identified by their ability to complement the function of effector domain mutants of HIV-1 Rev and visna virus Rev (10, 19). Leucines play a role in the function of atypical effector domains, but no core tetramer region can be identified (Fig. 1) (10, 19).

Our analysis identified a 17-amino-acid region of HTLV-1 Rex which can rescue the function of an effector domain mutant of HIV-1 Rev in a position-independent manner. This 17-amino-acid region can also act as a NES. Structural analysis of the NES of HTLV-1 Rex identified four leucines that are essential for activity. Further analysis illustrated that two of these leucines, those which are not part of the core tetramer, can functionally occupy more than one position. Our studies also revealed that the function of the effector domain is mediated by hydrophobic interactions since other hydrophobic amino acids can replace the essential leucines.

MATERIALS AND METHODS

Plasmids. The chloramphenicol acetyltransferase (CAT) reporter plasmids pDM128 and p138XRE have been previously reported (12, 13). The derivatives RevDE and RevE− have been previously described (12). The various fragments of HTLV-1 were generated by 25 to 30 cycles of PCR for fusion to Rev effector domain mutants. A *Bgl*II site in the reading frame A/GAT/CT$_n$ was added to

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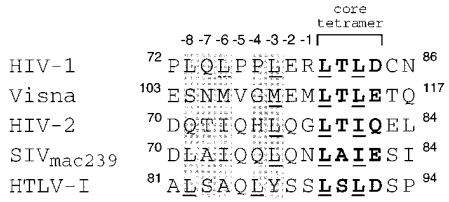
Typical effector domains



Atypical effector domains

FIG. 1. Alignment of typical and atypical effector domains. (A) Typical effector domains. The effector domain regions of HIV-1 Rev, visna virus Rev, HIV-2 Rev, SIV_{mac239} Rev, and HTLV-1 Rex are shown with the core tetramer regions in bold. Essential amino acids are underlined. Required for maximal function, leucine 74 of HIV-1 Rev is underlined with a dotted line. The important positions of this domain, positions −3, −4, and −6 through −8, are shaded. (B) Atypical effector domains. The effector domains of EIAV and FIV are shown. Atypical effector domains do not contain a recognizable core tetramer.

both ends of the fragment during amplification. Ten percent of the PCR product was isolated by agarose gel electrophoresis, purified with Geneclean (Bio 101), digested to completion with the appropriate restriction enzyme, precipitated, and ligated into the appropriate vector. All fragments were sequenced after insertion. A GCG clamp was present on the 5′ ends of all oligonucleotides to facilitate efficient restriction enzyme digestion. An additional 10 amino acids (DLWLT REPTA), including the Asp-Leu of the *Bgl*II site, are present at the C termini of all of the fusions to RevΔE and RevE−, with the exception of the amino acid 79 to 93 fragment which has a stop codon before the *Bgl*II site. Mutations in the context of HTLV-1 Rex were generated by Kunkel mutagenesis. A unique restriction site which had no effect on the protein coding sequence was typically included in the site-directed mutants to act as a genetic marker. All derivatives were confirmed by DNA sequencing. A new derivative of RevΔE was generated for the library of randomized mutants of the Rex effector domain. The polylinker GATCTCTGATCTAGAATCGATTAGCTAGCTAGTGATC was inserted into the *Bgl*II site of RevΔE to generate RevΔE/NI. The random library was generated by the synthesis of oligonucleotide gcgagatctaATGGACGCGnnnnnnGCT CAGnnnnnnAGTTCCTTATCCCTCGACTCCCCAtaatctagagcg, where n is a random nucleotide. Primers corresponding to the lowercase sequences on the ends of this oligonucleotide were used to amplify this pool of sequences by PCR. The amplified DNA was prepared as described above and digested with *Bgl*II and *Xba*I prior to cloning into RevΔE/NI to generate a library of mutants.

Cell culture and transfection. CV-1 cells were grown at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and grown in 5% CO_2. Cells were transfected by the standard protocol for $CaPO_4$-mediated transfection of adherent cells (26). A confluent plate of cells was split 1:8 approximately 24 h before transfection. Briefly, a total of 10 μg of plasmid DNA in 220 μl of 0.1× Tris-EDTA was mixed with 250 μl of 2× Hanks balanced salt solution. Thirty-one microliters of 2 M CaCL2 was added dropwise while the mixture was vortexed. The mixture was incubated at room temperature for 30 min before application to cells. For the transfection of CV-1 cells, the medium was removed and the $CaPO_4$-DNA mixture was applied to cells. After 15 min, 7 ml of medium was added to the plate. Approximately 48 h after transfection, cells were harvested with calcium- and magnesium-free phosphate-buffered saline (PBS) containing 5 mM EDTA. Harvested cells were used for quantitative β-galactosidase assays (ONPG [*o*-nitrophenyl-β-D-galactopyranoside]), and CAT assays were determined by thin-layer chromatography. Each transfection typically contained 2 μg of reporter DNA, 5 μg of transactivator DNA, 0.5 μg of pCH110 (β-galactosidase internal control), and pUC118 to a total of 10 μg. Cos7 cells were transfected with 20 μg of the appropriate plasmid to generate lysates for Western (immunoblot) analysis.

CAT assay. CAT assays were performed as previously described (13). All extracts were normalized for β-galactosidase activity before CAT analysis. All experiments were repeated at least three times. Similar results were obtained in repeated experiments. Percent conversion is the ratio of acetylated chloramphenicol divided by the total chloramphenicol in each reaction.

Generation of BSA-peptide conjugates. Four peptides with the following sequences were utilized in this study: the wild type, CMDALSAQLYSSLSLDSP; L90K, CMDALSAQLYSSKSLDSP; 14ββ, CMDAEFAQVFSSLSLDSP; and 36αα, CMDAVSAQVFSSLSLD. Bovine serum albumin (BSA)-peptide conjugates were generated by a modification of the protocol of Fischer et al. (9). Six milliliters of BSA (5 mg/ml) (Boehringer Mannheim) in PBS (pH 7.4) was incubated with 50 mg of sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (Pierce Chemical) for 1 h at room temperature. The BSA was subsequently separated from the unreacted sulfo-SMCC by chromatography over a G-50 Sephadex (fine) column (Pharmacia). Approximately 5 ml of the purified sulfo-SMCC-BSA (1 ml) was mixed with 1 ml of PBS (pH 6.5) containing 40% dimethyl sulfoxide and incubated with 5 mg of peptide overnight at 4°C. Then BSA-peptide conjugates were concentrated and separated from free peptides and dimethyl sulfoxide by three cycles of concentration and dilution with PBS (pH 7.4) in a Centricon C-10 unit (Amicon). Conjugation was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The altered mobility revealed that each BSA molecule contained approximately 15 to 20 peptides. The altered mobility was similar for each peptide used. Only a small fraction of cross-linked BSA was detected. The final concentration of each BSA-peptide conjugate was approximately 20 mg/ml. An aliquot of each derivative was labeled with *N*-hydroxysuccinimide (NHS)–fluorescein (Pierce Chemical), NHS-rhodamine (Pierce Chemical), or NHS-Cy3 (Amersham) in PBS (pH 7.4)–50% dimethyl sulfoxide for 2 h on ice. Then resulting labeled BSA-peptide conjugate was separated from the unincorporated label by G-50 Sephadex (equilibrated with PBS, pH 7.4) chromatography, with subsequent concentration by centrifugation in a Centricon C-10 unit.

Microinjection into Cos7 cells. Two days prior to injection, a confluent flask of Cos7 cells grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum was trypsinized, diluted 1:6 or 1:12, and seeded onto 25-mm-diameter glass coverslips. Cells were mounted into a custom temperature-controlled chamber and placed atop an inverted microscope equipped with microinjection and video monitoring equipment and a confocal scanning laser module (28). Approximately 50 fl of BSA-peptide conjugate was injected into the nuclei of Cos7 cells. During injections, cells were incubated with CO_2-deficient medium (Gibco). Most injections were performed at 20°C to slow the export process so that multiple groups of cells could be injected and monitored semisynchronously over a 30- to 40-min period. When injected at 37°C, most cells showed complete export of wild-type peptide conjugates within 1 or 2 min. Conservative operating conditions of the confocal microscope (low-level laser illumination and moderate- to high-level photomultiplier gain) were employed to minimize the photobleaching from laser illumination of two or three time points. Microinjection experiments with peptide conjugates were performed at least twice on separate days with their relevant controls.

Western analysis. Immunoblotting was performed as previously described (13). Lysates were separated on a 15% 1:30 bisacrylamide SDS-PAGE gel. Rev was detected by using a cocktail of three rabbit antisera specific to HIV-1 Rev. Two are anti-peptide antisera generated to amino acids 1 to 20 and 27 to 51 of HIV-1 Rev (kindly provided by M. Malim) and an antiserum generated against full-length HIV-1 Rev (kindly provided by M. Zapp). HTLV-1 Rex was detected with an anti-peptide antiserum directed against amino acids 80 to 102 (kindly provided by D. Derse).

Numbering of sequences. All the sequences and numbering presented here are based on the following GenBank sequences: HTLV, accession no. J02029; HIV-1, accession no. K03455; visna virus, accession no. M10608; HIV-2, accession no. M15390; SIV_{mac239}, accession no. M33262; FIV, accession no. M25729; and EIAV, accession no. M16575.

RESULTS

Identification of the minimal effector domain of HTLV-1 Rex. To initiate our characterization of the effector domain of HTLV-1 Rex, we sought to identify the minimal peptide that could complement the function of an effector domain deletion mutant of HIV-1 Rev (RevΔE). RevΔE consists of the first 78 amino acids of HIV-1 Rev. This region has been demonstrated to bind to the Rev-responsive element and oligomerize in a manner indistinguishable from that of wild-type HIV-1 Rev (33). To identify the minimal region, a series of smaller peptide-encoding fragments from the region of amino acids 66 to 118 of HTLV-1 Rex was generated. We have previously reported that this region of HTLV-1 Rex could fully complement the function of RevΔE (12). Each fragment generated by PCR was flanked by *Bgl*II sites to facilitate cloning in the appropriate reading frame into the RevΔE expression vector. The introduced *Bgl*II site added an Asp-Leu to both ends of the insert in each derivative. Nine amino acids were added to the C terminus before a stop codon in the vector was reached. These constructs were transiently transfected into CV-1 cells along with a specific CAT-based reporter, pDM128, which efficiently detects Rev function (13). This reporter system is derived from the second-intron region of HIV-1 expressed by the simian virus 40 immediate-early promoter. The CAT gene was inserted within the intron to occupy the normal position of the

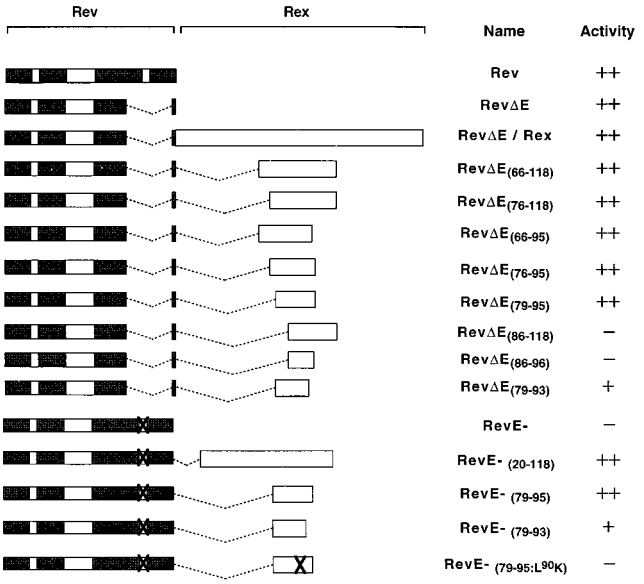


FIG. 2. Derivation of the minimal effector domain of HTLV-1 Rex. Schematics of plasmid derivatives are shown adjacent to the test results of their function in a Rev-specific assay (13). Rev sequences are shown as shaded bars, with white bands signifying the three domains of HIV-1 Rev (from left to right), the amino-terminal domain, the arginine-rich RNA binding domain, and the effector domain. RevΔE is shown with a deletion of the region from amino acids 79 to 114 of HIV-1 Rev, while RevE− is shown with a bold X through the effector domain. Deletion derivatives of the HTLV-1 Rex (open bars) were generated and fused to an effector domain deletion mutant, RevΔE, or an effector domain point mutant, RevE−. A mutant of the 79-95 region of HTLV-1 Rex was generated by changing the first leucine of the core tetramer to a lysine (L90K) and fused to RevE−. The name and corresponding activity (from none [−] to the highest [++]) of each derivative for pDM128 expression are shown on the right. The numbers of amino acids of HTLV-1 Rex are shown in parentheses.

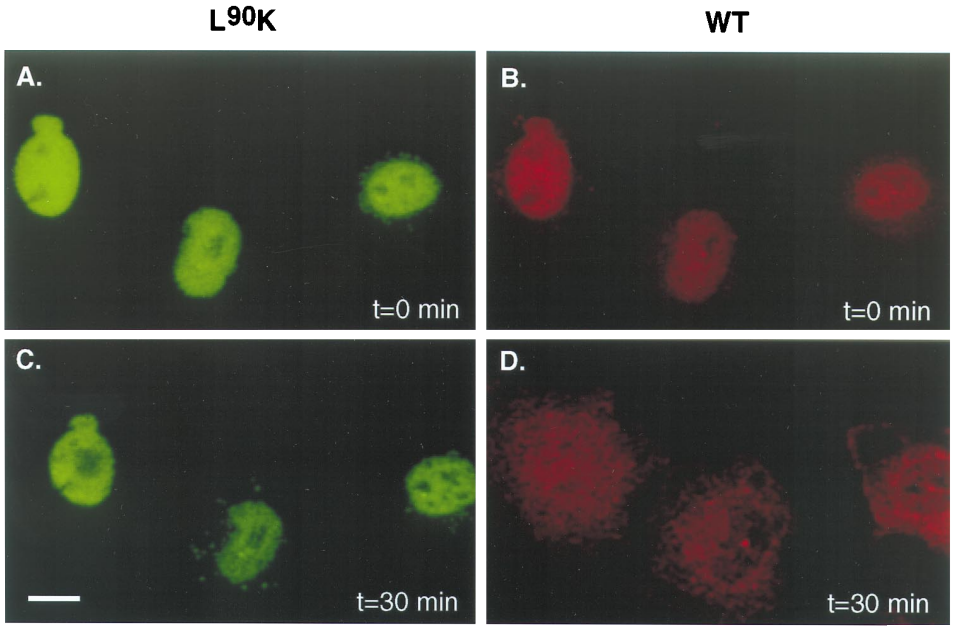
HIV-1 envelope. In the absence of facilitated RNA export, only spliced RNA in which the CAT gene has been deleted efficiently appears in the cytoplasm of transfected cells. If export of the unspliced RNA from the nucleus to the cytoplasm is facilitated, the CAT gene is efficiently expressed. All CAT assays were normalized to a cotransfected β-galactosidase expression plasmid.

This analysis, illustrated in Fig. 2, revealed that the fusion of amino acids 79 to 95 of HTLV-1 Rex contained sufficient information to complement a mutant of HIV-1 Rev containing a deletion in its effector domain. An overlapping fragment encoding amino acids 86 to 118 of HTLV-1 Rex was nonfunctional. A second derivative encompassing amino acids 86 to 95 was also nonfunctional. To further pare down the functional fragment, we generated a fragment encompassing amino acids 79 to 93 and including a stop codon directly after amino acid 93. This deleted the Asp-Leu and other amino acids on the C termini of the deletion derivatives tested (Fig. 2). The amino acids C-terminal to the second leucine in the core tetramer of the effector domain of HIV-1 Rev have been shown to be dispensable (23). The amino acid 79 to 93 fragment was able to restore RevΔE to levels that were approximately half of those of wild-type Rev.

The minimal effector domain of HTLV-1 Rex can complement effector domain mutations of HIV-1 Rev in a position-independent manner. To address the potential influence of the flanking amino acids of HIV-1 RevΔE on the function of the minimal Rex fragments, we tested the effects of position on the function of the minimal peptides. To this end, we tested the activities of the functional regions encompassing amino acids 20 to 118, 79 to 95, and 79 to 93 fused to the C terminus of an effector domain mutant of HIV-1 Rev (RevE−). This mutant was generated by altering three amino acids in the Rev effector domain and has been previously reported as DN2 (14). In this context, the Rex peptide is located at the C terminus of HIV-1 Rev, which is in contrast to the position occupied in RevΔE, a position roughly analogous to the native effector domain of Rev. The results of this analysis, shown in Fig. 2, revealed that these Rex effector domain fragments could complement, at similar levels, both a deletion and a point mutation of the HIV-1 Rev effector domain. The less efficient peptide, consisting of the region from amino acids 79 to 93 of HTLV-1 Rex, also showed equivalent activity in either context.

To demonstrate that the observed activity was specific to a functional Rex effector domain peptide, we created an inactive minimal Rex effector domain (amino acids 79 to 95) by changing a single amino acid in the minimal region's core tetramer. Thus, the first highly conserved leucine located at amino acid 90 of HTLV-1 Rex was modified to a lysine (L-90→K [L90K]). As shown in Fig. 2, this modification abrogated the ability of Rex to complement the function of RevE−. Western analysis indicated that all of the derivatives were efficiently expressed (data not shown). These results indicate that the Rev sequences flanking the complementing Rex peptide are not critical for function. Additionally, they confirmed that all of the information required for a fully functional effector domain is contained in the minimal peptide of amino acids 79 to 95 of HTLV-1 Rex.

The minimal effector domain of HTLV-1 Rex is a NES. Previous studies of the HIV-1 Rev effector domain (9) and a similar sequence in the cAMP-dependent protein kinase inhibitor (31) showed that these regions could mediate the export of molecules out of the nucleus. Because of the sequence similarity of the effector domains of HIV-1 Rev and HTLV-1 Rex, we wanted to determine if the HTLV-1 Rex effector domain could also act as a NES. Using the protocol of Fischer et al., we generated conjugates of BSA with a peptide consisting of the 17 amino acids encoding the minimal effector domain of HTLV-1 Rex (amino acids 79 to 95) or a mutant version containing the L90K mutation. If this region can act as a NES, it has the ability to facilitate the export of BSA conjugates after nuclear microinjection in somatic cells. In these experiments, conjugates with the wild-type peptide were labeled with rhodamine while conjugates labeled with fluorescein isothiocyanate (FITC) contained the L90K mutation. The two conjugates were mixed and microinjected into the nuclei of Cos7 cells. If export was specific, the rhodamine-labeled conjugates containing the wild-type peptide would be specifically exported to the cytoplasm while the FITC-labeled mutant conjugates would be retained in the nucleus. The mutant conjugates provide an essential control for nuclear integrity. As can be seen in Fig. 3, the conjugates containing the wild-type peptide were rapidly exported from the nuclei of microinjected cells (B and D) while the L90K conjugates were retained in the nuclei (A and C). The nuclear export of conjugates was also temperature dependent and saturable. When injected at 37°C, ~90% of wild-type conjugates were exported within 2 min, while cells injected at 20 to 22°C required 20 to 30 min to show the same extent of export (data not shown). The saturability of the export process was demonstrated by the reduced number of cells showing transport after injection with high concentrations of conjugates or by the competitive displacement by coinjection with high concentrations of unlabeled conjugates (not shown). To confirm that export was peptide specific and not a result of the fluorophore, we also tested conjugates that were reverse labeled. Hence, the injection of a mixture of FITC-



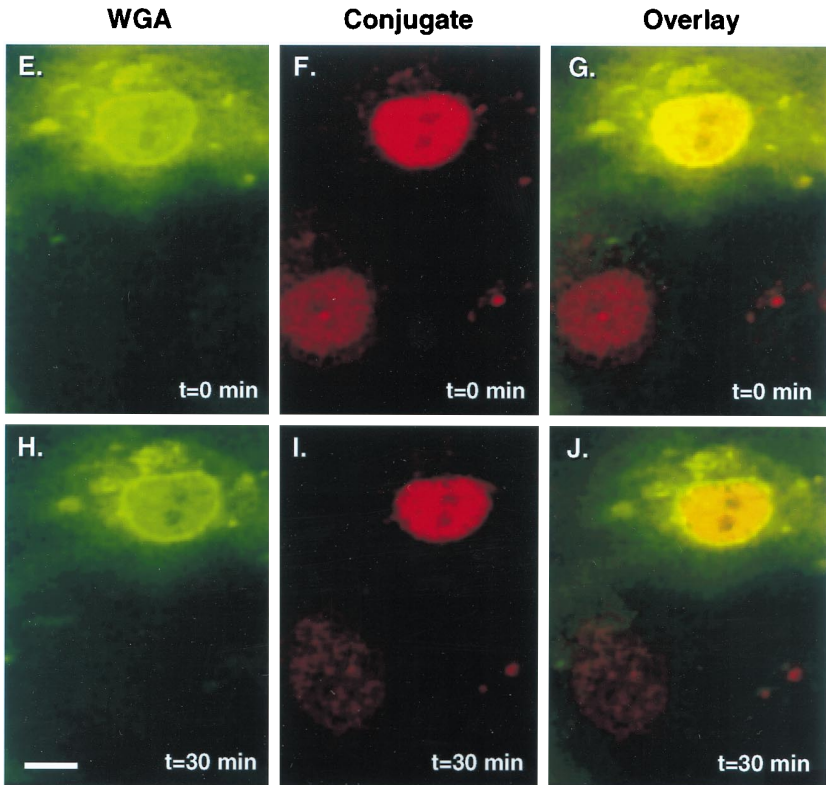


FIG. 3. The minimal effector domain is a NES which is inhibited by WGA. Fluorescently labeled conjugates of BSA and 17-amino-acid peptides representing the amino acid 79 to 95 region of HTLV-1 Rex, both wild-type and L90K mutant forms, were microinjected into Cos7 cells. Conjugates containing the wild-type peptide were labeled with Cy3 (red). L90K peptide conjugates were labeled with FITC (green). (A through D) Cells were injected into the nucleus with a 50-fl (28) mixture of wild-type and L90K conjugates at ~9 μg/ml (90-nM needle concentration). (A) Nuclear localization of L90K mutant conjugates after nuclear microinjection at time zero; (B) nuclear localization of wild-type conjugates at time zero in same field; (C) nuclear localization of mutant conjugates in same field after 30-min incubation at room temperature; (D) cytoplasmic localization of wild-type conjugates in same field after 30-min incubation at room temperature. (E through J) Inhibition of nuclear export by cytoplasmic preinjection with FITC-labeled WGA. The cell at the top of the field was injected with 100 fl of WGA (20 mg/ml) 30 min before nuclear microinjection of that cell and an adjacent cell (lower left) at time zero with Cy3 (red)-labeled wild-type peptide-BSA conjugates. (E) Localization of WGA at time zero, with specific staining of the nuclear envelope clearly visible; (F) localization of wild-type peptide-BSA conjugates at time zero; (G) overlay of the images in panels E and F; (H) localization of WGA at 30 min; (I) localization of wild-type conjugates at 30 min, with conjugates retained in the nucleus of the WGA-treated cell and normal export in the untreated cell (lower left); (J) overlay of the images in panels H and I. Bars, 5 μm.

labeled L90K conjugates and Cy3-labeled wild-type conjugates resulted in the export of only Cy3-labeled wild-type conjugates (data not shown).

It has previously been demonstrated that some types of transport through the nuclear pore can be inhibited by wheat germ agglutinin (WGA) (7), while other types are not affected (8). WGA can specifically block the export of mRNAs and small nuclear RNA (snRNA) through the nuclear pores of xenopus oocytes (5, 22). WGA specifically interacts with several proteins in the nuclear pore complex (6, 16). Our analysis indicates that the cytoplasmic preinjection of a somatic cell with WGA blocks the nuclear export mediated by the NES of Rex. The presence of WGA (labeled with FITC) completely blocked the export of Rex effector domain peptide-BSA conjugates (Cy3) from preinjected cells, while export was normal in an adjacent cell (Fig. 5E through J). This result is consistent with the Rex NES acting through a mechanism mediated by the nuclear pore complex. The possibility that WGA inhibits export by another mechanism cannot be excluded in this experiment.

Identification of four leucines required for the function of the effector domain of HTLV-1 Rex. We have previously shown that the leucines at positions 90 and 92, components of the core LXLX tetramer, are essential for HTLV-1 Rex function and conserved in many viral Rev-like proteins (12). Other studies have also shown that leucines are essential for both typical and atypical effector domain functions of viral Rev-like proteins (10, 18, 19). To further characterize the structure of the minimal effector region, we generated a series of substitution mutations spanning this region in the context of the full-size HTLV-1 Rex protein. Mutants with substitutions of glycine for two amino acids and encompassing the region of amino acids 81 to 94 of HTLV-1 Rex were generated by Kunkel mutagenesis. These mutants were compared with wild-type Rex for the ability to activate a specific CAT assay reporter for HTLV-1. This previously described assay utilizes p138XRE, a derivative of pDM138 containing the Rex-responsive element (12). The designations of all mutants described here show the wild-type amino acid followed by the number of its position in the protein and the mutant substitution. Therefore, A25G is the replacement of an alanine residue with a glycine at position 25. If two adjacent amino acids are mutated, both wild-type amino acids are shown first followed by their positions and the changes in the mutant (e.g., AL25,26GG).

All of the nonfunctional mutants removed essential leucine residues from the minimal effector region. As shown in Fig. 4A, while wild-type Rex and mutants SA83,84GG, YS87,88GG, and DS93,94GG were functional with similar activities, four of the mutants were completely nonfunctional; AL81,82GG, QL85,86GG, SL89,90GG, and SL91,92GG were all unable to transactivate the Rex-specific reporter in a transient-transfection assay. This analysis shows that the loss of any of the four leucines in the minimal region destroys its activity. Variations in activity were not the result of differential expression, since all mutants were expressed at levels similar to that of wild-type Rex (Fig. 4B). The functional mutant DS93,94GG had an altered mobility in repeated experiments. Altered mobilities for HTLV-1 Rex mutants have been previously observed and may be a consequence of altered posttranslational modification (3).

Two of the essential leucines can functionally occupy more than one position within this domain. To further determine the importance of the leucines at positions 82 and 86, we substituted the amino acid glycine at each position and tested these mutants for function. As illustrated in Fig. 5, these mutations abrogated effector domain function in the context of

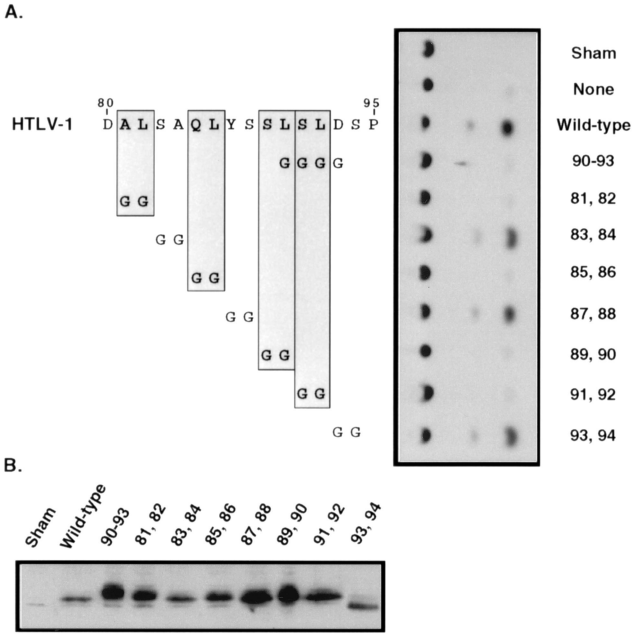


FIG. 4. Scanning mutagenesis of the HTLV-1 minimal effector domain. (A) Schematic illustration of scanning diglycine substitutions in the minimal effector domain region in the context of the full-size Rex protein. The specific changes in each derivative are shown under the wild-type sequence. The corresponding activity of each derivative on p138XRE is shown on the right. The position numbers of specific, modified amino acids are on the far right. Specific mutants affecting activity are shaded. Mutations changing any of the four leucines in this region abrogate function. (B) The equivalent expression of each mutant is shown by Western analysis.

native HTLV-1 Rex. When aligned by their core tetramers, the sequences of the effector domain regions of HTLV-1 Rex and HIV-1 Rev reveal that only the leucine at position 82 of Rex aligns with a leucine (at position 73 of HIV-1 Rev). There is an essential leucine at position 86 of Rex that is −4 relative to the core tetramer, whereas the essential leucine in Rev is at position −3 relative to the core tetramer. To explore the possibility that this essential leucine can occupy either the −3 or −4 position in this region, we generated the mutant LY86,87GL Rex. As shown in Fig. 5A, the LY86,87GL derivative was functional to a level that was approximately half that of wild-type Rex. We generated an additional mutant in which the leucine was placed at position −5 (QL85,86LG). As shown in Fig. 5A, this derivative was nonfunctional. These results indicate that a leucine at position 86 or 87 is required (−3 or −4 relative to the core tetramer) for HTLV-1 Rex to function.

We then explored the possibility that the leucine at position 82 of HTLV-1 Rex might also be functionally shifted. This is especially important because the leucine at position 73 was previously determined to be nonessential for HIV-1 Rev function. A mutation of the leucine at position 76 has been shown to decrease but not destroy HIV-1 Rev function (18). We generated a set of mutants of leucine 82 of HTLV-1 Rex in which a glycine was substituted for leucine 82 (−8 relative to the core tetramer) and a leucine was placed at positions −9, −7, and −6 relative to the core tetramer. As shown in Fig. 5A, when a leucine was present at position −7 in the context of L82G, the function of the mutant was equivalent to that of the wild type. A leucine at position −9 or −6 in the context of L82G was nonfunctional in the context of the native Rex protein. These results indicate that HTLV-1 Rex requires a leucine at position 82 or 83 (−8 or −7 relative to the core

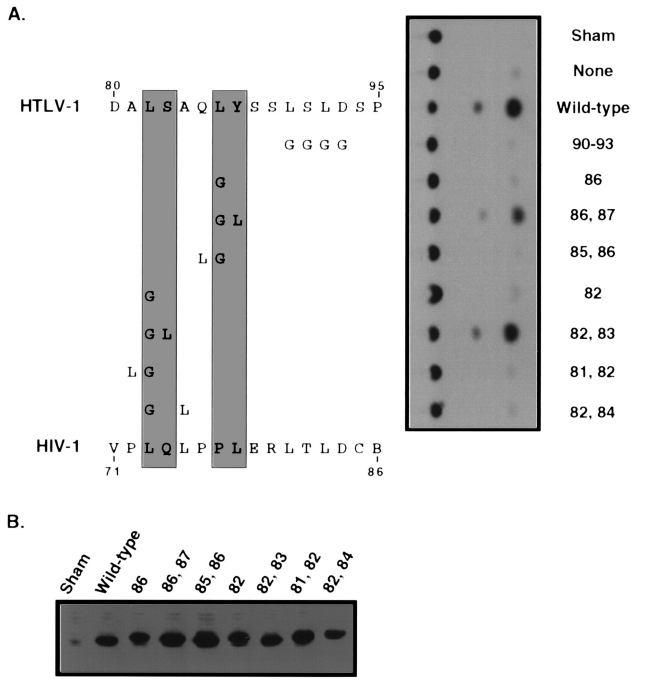


FIG. 5. Essential leucines can functionally occupy multiple positions in the HTLV-1 effector domain. (A) Schematic illustration of mutants designed to test the positional flexibility of essential leucines within the context of a full-size HTLV-1 Rex protein. The specific changes in each derivative are shown under the wild-type sequence. The sequence of the effector domain region of HIV-1 Rev is shown at bottom, and the corresponding activity of each derivative on p138XRE is shown on the right. The position numbers of modified amino acids are on the far right. Leucines are required at either position −3 or −4 and −7 or −8 for the function of HTLV-1 Rex. (B) The equivalent expression of each mutant is shown by Western analysis.

tetramer). The roughly equivalent expression of each derivative was demonstrated by Western analysis (Fig. 5B).

Identification of functional effector domains from a library of random amino acids at positions 82, 83, 86, and 87 of HTLV-1 Rex. The ability of the essential leucines of the effector domain to occupy more than one position and retain activity is a very surprising observation. To test the possibility of biases introduced by using the amino acid glycine, which has no side chain, and to further explore the basic structure of this domain, we generated a system to screen for functional derivatives from a pool of random mutants. To avoid problems associated with the tertiary structure of HTLV-1 Rex, we generated the random library in the context of the fully functional effector peptide encompassing amino acids 79 to 95.

To test the feasibility of screening a randomized library, we completed a series of pilot studies to determine our ability to identify functional derivatives in a mostly nonfunctional population. This is an important consideration because nonfunctional derivatives are dominant negative (14, 17). We determined that it was possible to efficiently detect a functional derivative in the presence of a fivefold excess of dominant negative miniprep DNA. These pilot studies also indicated that it was possible to screen pools of six colonies from our library by transfecting miniprep DNA and to identify functional derivatives.

An oligonucleotide of the sense strand of the amino acid 79 to 95 region was generated to include random nucleotides at the positions encoding amino acids 82, 83, 86, and 87, corresponding to positions −7, −8, −3, and −4 relative to the core tetramer. A stop codon was also placed directly after amino acid 95 of Rex. PCR primers were used to amplify the oligonucleotide population, which was inserted into a modified version of RevΔE, RevΔE/NI, that allowed directional cloning of the peptide-encoding fragments. A library was generated with the population of oligonucleotide-derived fragments. This library was screened by pooling six colonies from the library into 2-ml cultures with subsequent miniprep isolation of plasmid DNA by alkaline lysis. A total of approximately 750 colonies were tested by transient transfections of miniprep pools. The clones in any pool that appeared to be above background were individually tested in a second screen. A total of 12 clones that reconstituted activity above background levels were identified. Large-scale plasmid DNA for each clone was prepared and tested for the ability to transactivate the Rev-specific reporter pDM128. Four randomly selected nonfunctional clones were included in this analysis. Each functional clone had activity that was at least 10% above background. Four had wild-type activity, three had activity between 40 and 75% of that of the wild type, and five had activity levels between 10 and 40%. The sequences and activities of all 16 clones are illustrated in Fig. 6. Western analysis indicated that all of the identified clones were expressed to equivalent levels (data not shown). A hydrophobic amino acid was present in at least one of the two selected positions in the active derivatives. These results indicate that hydrophobic amino acids, in addition to leucine, can occupy important positions in the effector domain of HTLV-1 Rex.

To determine if the unique functional effector sequences identified in the random library could act as NESs, we synthesized peptides corresponding to the 14ββ and 36αα isolates. The 14ββ derivative restored the function of RevΔE to wild-type levels, while the 36αα isolate complemented function to about 60% of that of the wild-type sequence. Conjugates with BSA were generated as previously described. Then the conjugates were labeled with FITC, mixed with labeled conjugates containing the mutant peptide (L90K), and microinjected into the nuclei of Cos7 cells. As can be seen in Fig. 7, the 36αα peptide facilitated the nuclear export of fluorescently labeled BSA. Similar results were obtained with the 14ββ peptide (data not shown).

DISCUSSION

In this study, we have identified a minimal 17-amino-acid region of HTLV-1 Rex that complements effector domain mutants of HIV-1 Rev in a position-independent manner. This is a refinement of the previously identified 21-amino-acid region of HTLV-1 Rex which could restore the function of an effector domain mutant of HIV-1 Rev (30). Characterization of this region revealed a number of interesting features: (i) a bipartite consensus sequence composed of an LXLX core and an upstream region containing two essential leucines, (ii) hydrophobic amino acids can replace either of the upstream leucines, (iii) the positioning of the upstream hydrophobic amino acids is flexible, and (iv) the minimal 17-amino-acid region functions as a NES in living somatic cells. A suboptimally functioning 15-amino-acid peptide was also identified.

The export of BSA conjugated to the 17-amino-acid minimal effector domain region of HTLV-1 Rex confirms that it is a NES. This function has been demonstrated by direct nuclear microinjection of fluorescently labeled peptide-BSA conjugates. The function of the NES of HTLV-1 Rex is mediated through a temperature-sensitive saturable pathway, consistent with studies of the NESs of HIV-1 Rev and protein kinase inhibitor (9, 31). Further, we have demonstrated that the ex-

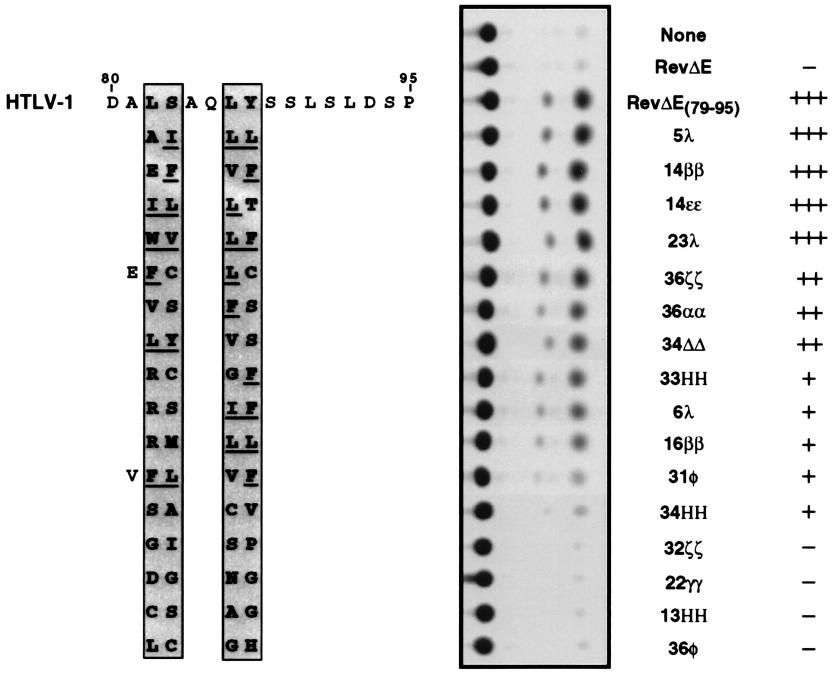


FIG. 6. Identification of functional effector domains from a library of random mutants. The diagramatic structures and activities of 12 clones with at least 10% wild-type function are shown. These clones were identified by screening a library of derivatives with random nucleotides at positions −3, −4, −7, and −8 in the 17-amino-acid minimal effector domain of HTLV-1 Rex. All derivatives were fused to RevΔE with a stop codon directly after amino acid P-95. Four nonfunctional derivatives randomly selected are also shown. The wild-type sequence of the amino acid 80 to 95 region of HTLV-1 Rex is shown at the top. The identified derivatives are ranked in descending order of activity. The sequence of each derivative is shown in shaded columns. The corresponding activities and names are on the right. Levels of function: 100 to 75% activity, +++; 40 to 75% activity, ++; 10 to 40% activity, +; >10% activity, −.

port mediated by this class of NESs is inhibited by the presence of WGA. This observation is consistent with a model in which the export mediated by this class of NESs is a result of specific interactions with components of the nuclear pore complex.

Recently, competition experiments have shown that snRNAs and 5S RNA utilize the same pathway of nuclear export as do the Rev-like proteins (9). In that study by Fischer et al., the injection of large amounts of BSA conjugates containing the effector domain of HIV-1 Rev blocked the export of snRNAs and 5S RNA but had no effect on the export of mRNA, tRNAs, or rRNAs. Our observation that the Rex NES pathway is WGA sensitive is in agreement with the previous observation that WGA can block the nuclear export of snRNAs injected into the nuclei of xenopus oocytes. It remains to be determined if the NES recently identified in heterogenous nuclear RNPA1 acts through a similar or distinct pathway than that utilized by the NESs of Rev-like proteins (21).

The screening of the random library revealed that hydrophobic amino acids could substitute for the upstream leucines within the minimal 17-amino-acid region. This analysis identified four sequences that encoded wild-type activity, and all contained hydrophobic residues at positions −3 or −4 and −7 or −8 upstream of the LXLX core. Only a single charged residue is present in the 16 randomly generated amino acids in these four wild-type peptides. The hydrophobic amino acids phenylalanine and tryptophan, in addition to leucine and isoleucine, were able to restore the full activity of the peptide. All the peptides with either maximal or intermediate activity conform to the consensus sequence (Fig. 1) if related hydrophobic amino acids can replace leucines. Similarly, leucines in a leucine zipper can be functionally replaced by chemically related hydrophobic amino acids (15).

The studies presented here generate a unique type of flexible consensus sequence for the NESs of Rev-like proteins. The functional interactions involved in the activity of the NES is mediated by hydrophobic amino acids. An alignment of the typical effector domains from several Rev-like proteins (Fig. 1) reveals the core tetramer and required hydrophobic amino acids at positions −3 or −4 and −6, −7, or −8 relative to the core tetramer. Therefore, the NESs of Rev-like proteins have a flexible consensus sequence because essential amino acids can functionally occupy multiple positions within a specific region. This consensus sequence is a hydrophobic amino acid at position −3 or 4 relative to the core tetramer and a second hydrophobic amino acid at position −3 or 4 relative to the first upstream leucine for efficient activity. A spacing of 5 amino acids between the two upstream leucines is partially functional, as seen with the LY86,87GL mutant (Fig. 7), whereas separating them by 2 amino acids is nonfunctional, as seen with the L82G,A84L mutant (Fig. 7). All of the leucine-rich effector domains shown in Fig. 1 fit this exotic consensus sequence. Further analysis needs to be performed in order to completely test this proposed consensus sequence for the typical effectors of Rev-like proteins. It is interesting that a derivative similar to our LS82,83GL mutant has previously been reported (3). It was determined that the M12 mutant (LS82,83DL) in the study by Böhnlein et al. (3) did not affect HTLV-1 Rex function; therefore, the importance of the leucine at position 82 was missed.

The relationship between atypical and typical effector domains remains to be determined. It is compelling to speculate that they are structurally related. Both typical and atypical effectors contain essential hydrophobic amino acids. Further, the atypical effector domain of EIAV and the typical effectors of HIV-1, HTLV-1 Rex, and visna virus have recently been demonstrated to interact with the same cellular factor. This

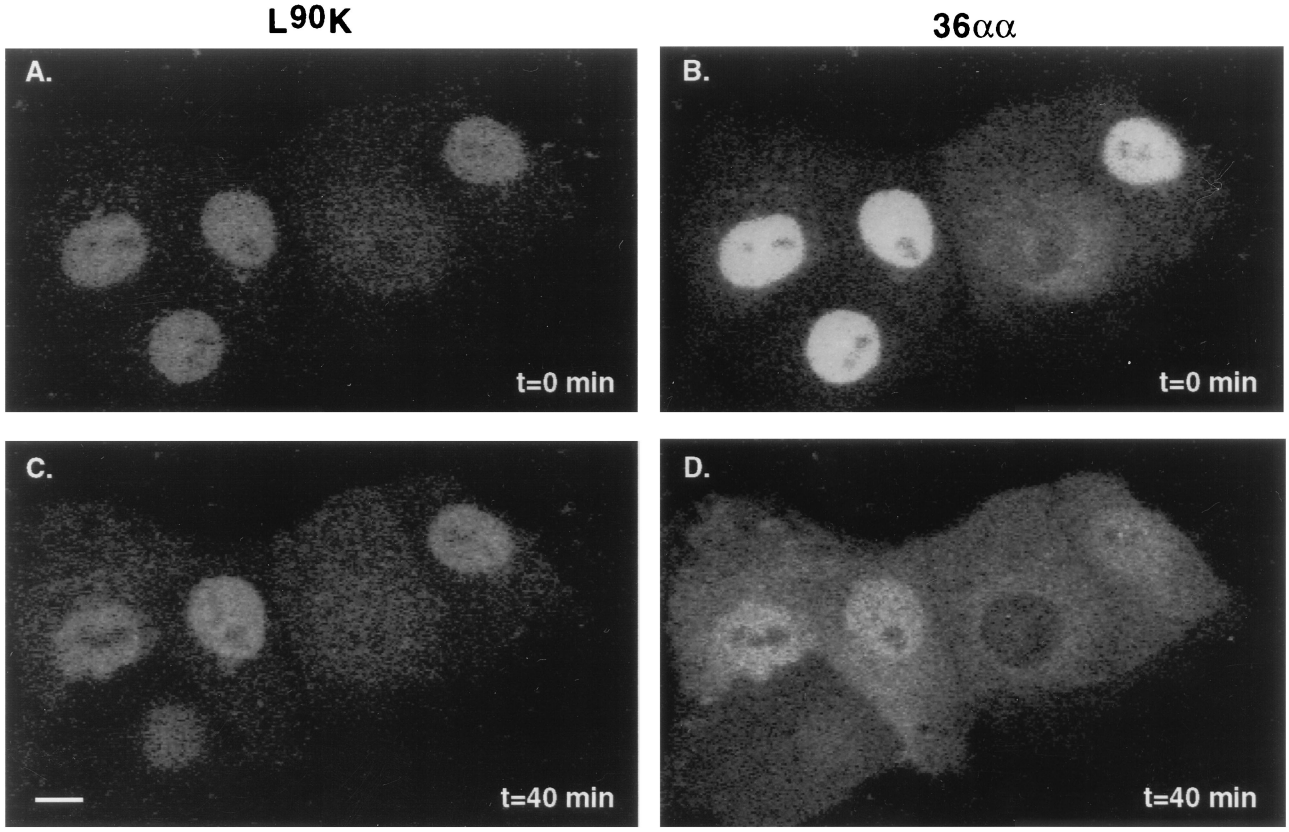


FIG. 7. The functional 14ββ and 36αα derivatives can function as NESs. Peptides corresponding to the 14ββ and 36αα derivatives were generated, conjugated with BSA, and labeled with FITC. 14ββ and 36αα conjugates were mixed with rhodamine-labeled L90K mutant conjugates and microinjected into Cos7 cells at 9 μg/ml. (A) Localization of mutant conjugates in a field of five microinjected cells at time zero. The second cell from the right was inadvertently microinjected in the cytoplasm, while the rest were nuclear injections. (B) Localization of 36αα conjugates in same field at time zero. (C) Nuclear localization of L90K mutant conjugates 40 min after injection. (D) Cytoplasmic localization of 36αα conjugates 40 min after microinjection. Similar results were seen for the 14ββ conjugates (data not shown). Bar, 5 μm.

cellular factor, called Rab or hRip, has nucleoporin-like characteristics and is an excellent candidate to be the cellular factor which mediates the export of the NESs of Rev-like proteins (2, 11).

This high level of variability among NESs and effector domains with equivalent activities suggests that the secondary structure of the NES is unconventional. This characteristic of variable positioning of functional hydrophobic amino acids suggests that the effector domain does not form a common secondary structure, such as an alpha-helix or a beta pleated sheet, in which shifting the location of an important residue by one position has a great effect upon a domain's three-dimensional structure. The position independence of the 17-amino-acid minimal effector domain peptide makes it a good candidate for physical studies to determine the structure of this class of NESs.

The inherent flexibility of the NES of HTLV-1 Rex is reminiscent of the acidic activation domains of transcription factors and nuclear import signals (NLS). In these domains, the overall amino acid character, either charge or hydrophobicity, is as important as the specific spacing of essential residues. This is in contrast to the other types of domains involved in protein-protein interactions, such as the leucine zipper, in which essential amino acids can be replaced with chemically related residues but the spacing is rigidly conserved.

It is interesting to note the similarities among the known systems for transport through the nuclear pore complex. The NES is analogous to the karyophilic NLS in several ways. The functions of the karyophilic NLS are mediated by salt bridges, utilizing the positively charged amino acids lysine and arginine, while the NES function is mediated by specific hydrophobic interactions, utilizing the amino acids leucine, isoleucine, phenylalanine, methionine, cysteine, tryptophan, and valine. The positions of functionally important amino acids are variable in both the NLS (reviewed in reference 4) and NES. This is demonstrated by a second class of NLS which have a bipartite structure. The NLS of nucleoplasmin (25), nucleolin (27), the retinoblastoma gene product (32), and a number of other nuclear proteins consist of two groups of arginine and lysine separated by a variable-size spacer of random amino acids. In the bipartite NLS, both regions of positive charge are required for activity. We have demonstrated the great potential for functional variation in our characterization of the NES of HTLV-1 Rex. The potential for variation may allow these important localization domains to be more easily placed within the primary sequences of proteins without disrupting the overall structure and function.

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