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Nuclear Transport of Human Immunodeficiency Virus Type 1, Visna Virus, and Equine Infectious Anemia Virus Rev Proteins: Identification of a Family of Transferable Nuclear Export Signals

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The human immunodeficiency virus type 1 Rev *trans* activator binds directly to unspliced viral mRNA in the nucleus and activates its transport to the cytoplasm. In addition to the sequences that confer RNA binding and nuclear localization, Rev has a carboxy-terminal region, the activation domain, whose integrity is essential for biological activity. Because it has been established that Rev constitutively exits and reenters the nucleus and that the activation domain is required for nuclear exit, it has been proposed that Rev's activation domain is a nuclear export signal (NES). Here, we used microinjection-based assays to demonstrate that the activation domain of human immunodeficiency virus type 1 Rev imparts rapid nuclear export after its transfer to heterologous substrates. NES-mediated export is specific, as it is sensitive both to inactivation by missense mutation and to selective inhibition by an excess of the wild-type, but not mutant, activation domain peptide. Examination of the Rev *trans* activators of two nonprimate lentiviruses, visna virus and equine infectious anemia virus, revealed that their activation domains are also potent NESs. Taken together, these data demonstrate that nuclear export can be determined by positively acting peptide motifs, namely, NESs, and suggest that Rev proteins activate viral RNA transport by providing export ribonucleoproteins with specific information that targets them to the cytoplasm.

The trafficking of molecules between the nucleus and the cytoplasm occurs through nuclear pores (19, 28). These complex channeled structures comprise upwards of 1,000 proteins and allow molecules to traverse the nuclear membrane in either direction by active (signal-mediated) transport as well as by passive diffusion (15, 33, 50, 90). Given that proteins residing in the nucleus must relocate from their site of synthesis in the cytoplasm and cytoplasmic RNAs transcribed in the nucleus must be exported from the nucleus, there has long been much scientific interest in the mechanisms responsible for the translocation of macromolecules through nuclear pores.

Numerous details of protein nuclear import are now understood, and a unifying model that accommodates the findings of many laboratories can be envisioned (40, 74, 80). Proteins destined for nuclear import harbor sequences, known as nuclear localization signals (NLSs), that typically consist of one or two stretches of basic amino acids (16, 37). The first step of import requires that the NLS interacts with the α subunit of a cytoplasmic complex, known as karyopherin or importin (1, 11, 39, 40, 80, 83, 108). Then a second subunit, termed karyopherin (or importin) β, mediates the docking of this complex with the nuclear pore (10, 40, 86). Finally, the NLS-containing protein is translocated through the pore by an energy-dependent process that requires the Ran GTPase (75, 78). However, it has already been established that protein import is more complex than this, as both a Ran-interacting protein, B-2 (79), and heat shock cognate 70 (54, 97) are known to be important for efficient NLS-dependent import.

Much less is currently understood regarding the processes by which RNA exits the nucleus (56). One important consideration in RNA export is that naked RNA is not the substrate for export; it is as complexes with proteins, termed ribonucleoproteins (RNPs), that RNA is transported to the cytoplasm (53, 61, 73). Accordingly, it seems likely that the determinants of RNP export could reside within RNA itself, for example, at the monomethylated (m^7G) guanosine 5′ cap of mRNA and some U small nuclear RNAs (45), the proteins, or a combination of both. As with NLS-directed import, a variety of proteins have been shown or implicated as being important for RNA export. Some of these can bind directly to the RNAs destined for export (32, 42, 55, 84, 92), whereas others, such as Ran (58, 94) and components of the pore itself (3, 18, 23, 41), are likely to play more general roles. By analogy with protein import, it can be postulated that some of these RNA-binding proteins function in a manner similar to that of karyopherin α by targeting specific RNPs for export, whereas more general factors act at subsequent steps in the export process. In light of this viewpoint, it is interesting that a number of RNA-binding proteins are able to traverse the nuclear membrane in a nucleus-to-cytoplasm direction; examples include nucleolin (6, 95), the heterogeneous nuclear RNP particle A1 protein (84), the U1 small nuclear RNP U1A protein (60), and the ribosomal L5 protein and TFIIIA (42). Indeed, it has previously been suggested that the nuclear export of heterogeneous nuclear RNP A1, L5, and TFIIIA is, in each case, involved in the transport of particular RNPs to the cytoplasm. Accordingly, if RNA export is to be fully understood, it will be important to determine how RNA-binding proteins that exit the nucleus are able to do so. To date, there is very little information in this area, and it remains controversial as to whether protein export is governed by specific *cis*-acting sequences (export signals) (72, 77), by the extent of nuclear retention (95), or alternatively, as a consequence of being bound to exported RNA.

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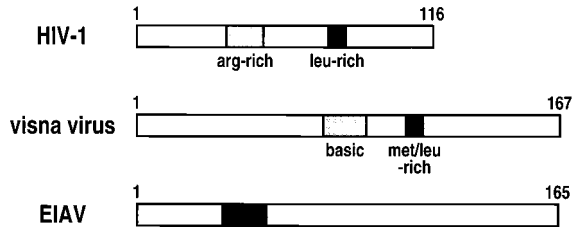

FIG. 1. Domain organizations of lentivirus Rev proteins. Activation domains (black boxes) have been defined in all three proteins, whereas RNA binding domains (gray boxes) that are rich in basic residues have been identified only in the HIV-1- and visna virus-encoded proteins.

When evaluating the contribution of RNA-binding proteins to RNA export, it is valuable to consider the role of the human immunodeficiency virus type 1 (HIV-1) Rev *trans* activator in viral mRNA expression (12). HIV-1 generates the array of fully spliced (~2-kb), incompletely spliced (~4-kb), and unspliced mRNAs required for expression of the full complement of viral proteins from a single ~9-kb primary transcript by exploiting both inefficient and alternative splicing (81, 85, 96). Because they contain functional introns, the 9- and 4-kb transcripts are confined, in the absence of Rev, to the nucleus, where they are either degraded or spliced to completion (27, 68). Rev, which is itself encoded by a subset of the 2-kb transcripts, therefore acts by sparing these transcripts from nuclear turnover and activating their transport to the cytoplasm (8, 20, 27, 46, 51, 69). Once in the cytoplasm, the 9- and 4-kb transcripts serve as templates for virion protein synthesis and, in the case of the 9-kb RNA, as the genomic RNA of newly assembled viral particles (26, 98).

The Rev protein of HIV-1 is modular in structure and contains two distinct and autonomous functional domains (Fig. 1). Toward the amino terminus is a region rich in arginine residues that confers nuclear and nucleolar localization as well as viral RNA binding on Rev (5, 13, 51, 66, 67, 82, 109, 110). The RNA target to which Rev binds is an elaborate stem-loop structure, the Rev response element (RRE), that is found within the *env* region of all 9- and 4-kb HIV-1 transcripts (44, 69, 89). Inhibiting this interaction by disrupting either the RRE or the RNA binding domain of Rev results in the abrogation of Rev-activated RNA export. Toward the carboxy terminus is a smaller domain of nine amino acids that contains four closely spaced leucine residues (see Fig. 2A) and is also essential for Rev function (52, 66, 70, 76, 82, 103). Because this region, termed the activation (or effector) domain, does not participate in RNA binding or nuclear localization, it is believed that it targets bound viral RNAs for export by interacting with host cell proteins (cofactors). Interestingly, it has recently been demonstrated that although it appears to be localized to the nucleus, Rev actually shuttles constitutively between the nucleus and the cytoplasm (59, 77, 87). Of particular significance, however, was the finding that functional inactivation of the activation domain always resulted in the inability of the mutant protein to exit the nucleus (77, 107). On the basis of the correlation between Rev export and RNA export, it has been hypothesized that this protein not only activates the nuclear export of unspliced viral RNA but is itself an integral component of exported RNPs.

HIV-1 is not the only retrovirus to encode a Rev protein. In fact, all of the lentiviruses (of which HIV-1 is the most extensively studied) as well as the human T-cell leukemia viruses appear to express functionally analogous proteins (48, 49, 62, 65, 93, 99, 101). The Rev protein of the ovine lentivirus visna virus possesses a domain organization highly reminiscent of HIV-1 Rev (Fig. 1) (102); it has a highly basic RNA binding domain and a carboxy-terminal activation domain that contains evenly spaced hydrophobic residues critical for function (see Fig. 6A). In contrast, the structure of equine infectious anemia virus (EIAV) Rev is less well-defined, and only the activation domain, which is somewhat larger and different in character to those of HIV-1 and visna virus, has been mapped (Fig. 1) (see Fig. 6A also) (35, 71). Importantly, it has been shown that the activation domains of HIV-1, visna virus, and EIAV Rev proteins are functionally autonomous in that each can be appended to the RNA binding domains of heterologous Rev proteins to create chimeras that activate RNA export with the substrate specificity of that particular RNA binding domain (35, 71, 102). Not surprisingly, therefore, it is believed that all Rev proteins have a common mechanism of action.

A prediction which can be made from the finding that activation domain integrity is essential for HIV-1 Rev's translocation to the cytoplasm is that the activation domain is a nuclear export signal (NES). However, it can also be argued that export is merely a consequence of being bound to RNA and that the signals which specify export reside elsewhere within the RNP. To discriminate between these possibilities, we transferred the activation domains of the HIV-1, visna virus, and EIAV Rev proteins to heterologous substrates and evaluated their respective capacities to confer nuclear export after microinjection into the nuclei of somatic cells. Here, we demonstrate that all three wild-type activation domains serve as NESs and that nonfunctional variants of each do not. These data are therefore consistent with the notion that lentivirus Rev *trans* activators select viral mRNAs for transport by binding to those RNAs and providing export RNPs with a signal which is recognized by the host cell's nuclear export machinery. Importantly, these findings provide strong support for the concept that a specific peptide motif can act as an NES.

MATERIALS AND METHODS

Molecular clones. Eukaryotic expression vectors that encode the wild-type *rev trans* activator genes of HIV-1, visna virus, and EIAV have previously been described (35, 69, 101). The following derivative versions of these vectors in which activation domain function has been abolished by missense mutagenesis of critical leucine residues have also previously been described: for HIV-1, M10 (Leu-78–Glu-79→Asp-Leu) and M29 (Leu-83→Ala) (see Fig. 2A) (66, 70); for visna virus, ΔAD (Leu-112–Thr-113–Leu-114→Ala-Ser-Ser) (see Fig. 6A) (102); and for EIAV, ΔAD (Leu-49→Ala) (see Fig. 6A) (35).

Isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible glutathione *S*-transferase (GST)-Rev fusion protein expression vectors were generated by standard approaches. Specifically, regions of wild-type and mutant *rev* genes that span the respective activation domains were amplified by PCR and inserted into the polylinker of pGEX-2T (Pharmacia Biotech, Inc., Piscataway, N.J.) so that a hinge of two glycine residues was always introduced between the GST and Rev moieties. These *rev* coding regions correspond to residues 67 to 116 of HIV-1 Rev (hRev), residues 101 to 167 of visna virus Rev (vRev), and residues 27 to 59 of EIAV Rev (eRev). In the cases of hRev and vRev, these regions are clearly distinct from the domains that are rich in basic amino acids and are known to be involved in binding to RNA (5, 66, 67, 82, 102, 110). The nomenclature of the fusion proteins encoded by these vectors reflects the origin of the *rev* sequences and whether the gene itself was wild type or mutated; for example, GST: vRev/WT harbors the wild-type activation domain of visna virus Rev.

To facilitate the immunological detection of vRev and eRev fusion proteins, the relevant expression vectors were modified at the 3′ termini of the *rev* inserts by inclusion of a synthetic sequence that encodes the epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) recognized by the Myc-specific monoclonal antibody 9E10 (see Fig. 6) (22).

Purification of GST proteins. All GST-containing proteins were purified by standard procedures in the absence of any detergent. Briefly, expression vectors were introduced into *Escherichia coli* BL21 and bacteria were grown in ampicillin-containing medium to an optical density at 600 nm of ~0.6; at this time, expression was induced for 2 h with 100 μM IPTG. After being harvested, cells were resuspended in phosphate-buffered saline (PBS) supplemented with 250 μM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per ml, and 2 μg of leupeptin per ml and disrupted by sonication, and lysates were cleared by centrif-

ugation at 5,000 × *g* for 5 min in a microcentrifuge. Glutathione-Sepharose 4B (Pharmacia Biotech, Inc.) was added to lysates, beads were washed thoroughly with PBS, and bound proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0). Eluates were dialyzed against PBS, concentrated, and stored at −20°C. Protein concentrations were determined by the method of Bradford (7).

Western (immunoblot) analyses. Samples (~100 ng) of each purified GST-containing protein were resolved on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and electroeluted onto nitrocellulose filters. Detection was accomplished by initial hybridization with murine monoclonal antibodies specific for GST (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) (all proteins), the carboxy terminus of hRev (47), or the Myc epitope (vRev and eRev proteins) (22). Then bound antibodies were visualized by secondary hybridization with a horseradish peroxidase-conjugated anti-mouse antibody raised in goats (Fisher-Biotech, Pittsburgh, Pa.), enhanced chemiluminescence (Amersham Corp., Arlington Heights, Ill.), and autoradiography.

Peptide synthesis, covalent cross-linking, and biotinylation. Peptides of 15 residues harboring the wild-type and M10 versions of the HIV-1 Rev activation domain were synthesized by using an automated Applied Biosystems 430A peptide synthesizer. The sequence of the wild-type peptide was Cys-Gly-Gln-Leu-Pro-Pro-Leu-Glu-Arg-Leu-Thr-Leu-Asp-Ser-Asn; the two residues altered in the M10 peptide are underlined. To ensure that each peptide would be cross-linked efficiently via its amino-terminal residue, a cysteine residue was incorporated at position 1 and the naturally occurring cysteine at position 14 was replaced with a serine residue.

Peptides were covalently coupled to bovine serum albumin (BSA; a protein with a relative molecular mass of ~68 kDa) by using the heterobifunctional cross-linker *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce, Rockford, Ill.) according to the manufacturer's instructions. After cross-linking, BSA-hRev and BSA-M10 conjugates were dialyzed against PBS, concentrated, and stored at −20°C. The average number of peptides coupled to each BSA molecule was estimated to be approximately 10, as judged by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. BSA, BSA-hRev, and BSA-M10 were biotinylated by using sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (Pierce) according to the manufacturer's instructions, dialyzed against PBS, concentrated, and stored at −20°C.

Cell culture, microinjection, and indirect immunofluorescence. HeLa cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. Two days prior to microinjection, cells were seeded onto glass coverslips at a density of 5×10^5 cells per 60-mm-diameter dish. GST fusion proteins and BSA conjugates were injected at final concentrations of 6 to 8 mg/ml by using a microinjector system (Eppendorf, Hamburg, Germany) and returned to the required temperature. Importantly, each protein preparation was supplemented with 5 to 6 mg of rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) so that the site of injection could be determined. At various times after injection, cells were fixed with 3% paraformaldehyde and the subcellular localization of injected proteins was determined by double-label indirect immunofluorescence (77). GST-containing proteins were detected by hybridization with anti-GST, anti-hRev, or anti-Myc monoclonal antibodies and subsequently with a goat anti-mouse antibody conjugated to Texas red (TXRD) (FisherBiotech); IgG was visualized with a donkey anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, Inc.). Biotinylated BSA molecules were detected in a single step by hybridization with TXRD-conjugated streptavidin (FisherBiotech). Samples were observed by epifluorescence with a Nikon Microphot-FXA microscope at a magnification of ×400.

RESULTS

One of the principal objectives of these experiments was to determine whether the activation domains of lentivirus Rev *trans* activators can serve as autonomous NESs. On the basis of previous studies in which the Rev protein of HIV-1 was shown to exit the nucleus in an activation domain-dependent fashion, it has been suggested that this relatively short sequence of nine amino acids constitutes an NES (77). However, to formally prove that a protein sequence can function as an NES, it is necessary to demonstrate that nuclear export capability can be acquired after the transfer of that sequence onto a heterologous substrate.

The activation domain of the HIV-1 Rev protein is a transferable NES. To address nuclear export experimentally, the molecule being examined must first be expressed in the nucleus. In terms of proteins, this can be achieved in one of two ways. First, the protein can contain an NLS so that it naturally accumulates in the nucleus. Under these circumstances, the nuclear export of a given protein could, potentially, be detected by its migration from one nucleus to another in the context of a heterokaryon (6, 43, 77, 84, 95) or, alternatively, by its ability to transport bound molecules such as IgGs to the cytoplasm (59). A second approach would be to introduce a protein that lacks an NLS directly into the nucleus by microinjection and then to score export as time-dependent relocalization to the cytoplasm (17, 25, 43, 72, 104). Because this latter strategy does not require that the protein harbor an NLS, it offers the advantage of being able to segregate nuclear export from the reverse process, namely, import back into the nucleus.

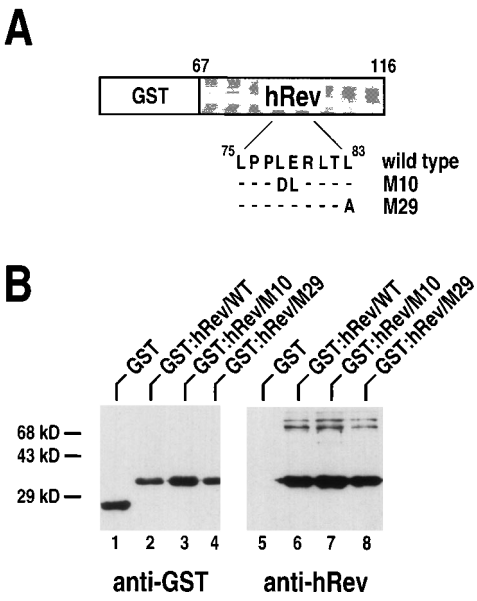

FIG. 2. Structure and purification of GST:HIV-1 Rev fusion proteins. (A) The transferable activation domains (residues 67 to 116) of wild-type HIV-1 Rev protein and nonfunctional M10 and M29 mutants were fused in frame to the carboxy terminus of GST. The primary amino acid sequence of the defined minimal activation domain of the wild-type protein, along with the alteration(s) present in each missense mutation, is shown; a dash indicates that the amino acid is unchanged. (B) Western analysis of affinity-purified unit-length GST (lanes 1 and 5), GST:hRev/WT (lanes 2 and 6), GST:hRev/M10 (lanes 3 and 7), and GST:hRev/M29 (lanes 4 and 8) fusion proteins. Filters were initially hybridized with murine monoclonal antibodies specific for GST (lanes 1 to 4) or the carboxy terminus of hRev (lanes 5 to 8). The positions of prestained protein molecular mass (in kilodaltons [kD]) standards are indicated on the left.

On the basis of these considerations, we chose to examine the ability of the hRev activation domain to function as an NES by covalently attaching it to heterologous proteins and then microinjecting these into the nuclei of somatic cells. In the first series of experiments, we fused the carboxy-terminal region of hRev (residues 67 to 116) to the carboxy terminus of GST (Fig. 2A). Importantly, this region does not harbor the arginine-rich domain (residues 35 to 50) that mediates both the localization of Rev to the nucleus and its binding to RNA (5, 66, 67, 82, 110). The absence of these particular sequences from these GST fusion proteins was considered especially advantageous, as it has been suggested that the nuclear export of RNA-binding proteins is driven by RNA export itself. In addition to the wild-type activation domain, the analogous regions of nonfunctional mutants M10 and M29 (66, 70) were also fused to the carboxy terminus of GST (Fig. 2A). These three GST:hRev proteins, as well as GST itself, were purified by affinity chromatography and assessed for integrity by Western blotting (Fig. 2B). As expected, each full-length and intact ~32-kDa GST:hRev fusion was readily detected with antibodies specific

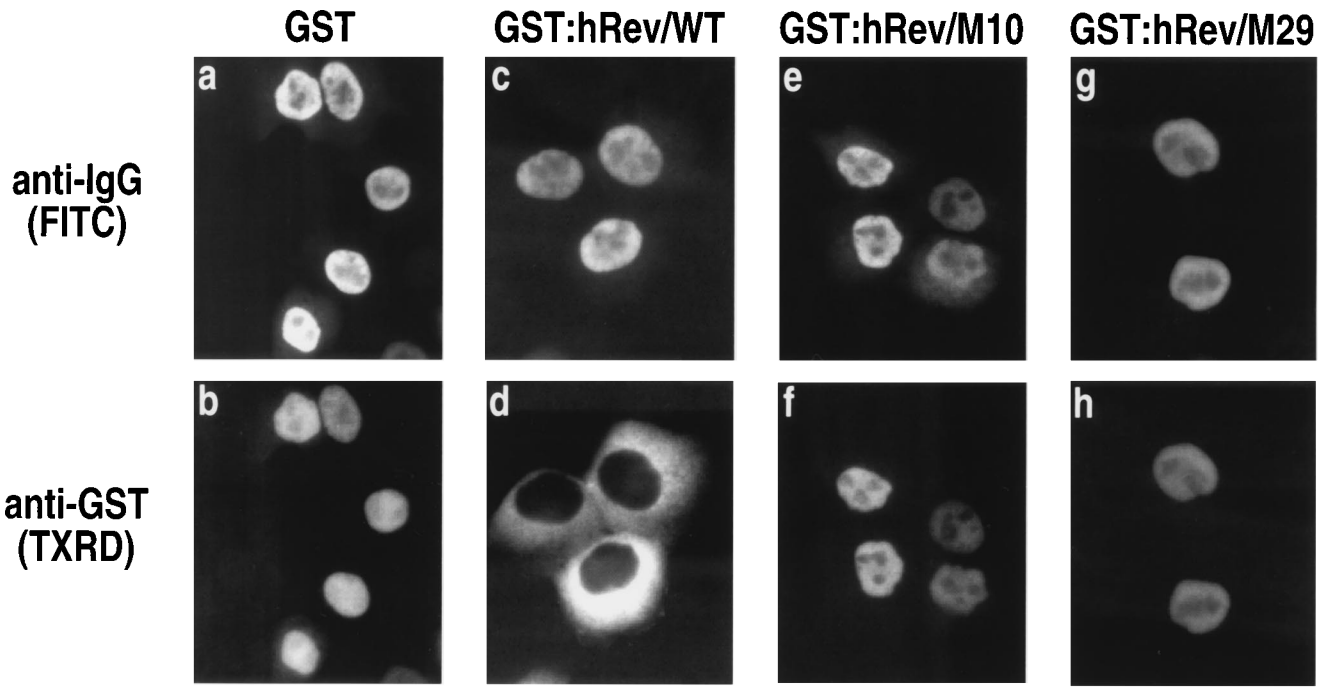

FIG. 3. The activation domain of the HIV-1 Rev *trans* activator is a potent NES. HeLa cells were injected into the nucleus with mixtures of rabbit IgG and GST (a and b), GST:hRev/WT (c and d), GST:hRev/M10 (e and f), or GST:hRev/M29 (g and h). After incubation for 30 min at 37°C, cells were fixed and subjected to double-label indirect immunofluorescence. IgG was visualized in a single step with a donkey anti-rabbit antibody conjugated to FITC (a, c, e, and g), whereas GST and GST:hRev fusions were detected in two steps with a GST-specific monoclonal antibody followed by a goat anti-mouse antibody conjugated to TXRD (b, d, f, and h). Samples were viewed at a magnification of ×400.

for GST (Fig. 2B, lanes 2 to 4) or the carboxy terminus of Rev (lanes 6 to 8).

The nucleocytoplasmic trafficking phenotypes of the GST and GST:hRev proteins were determined by microinjecting them into the nuclei of living HeLa cells. After injection, cells were incubated at 37°C for 30 min, fixed, permeabilized, and then subjected to immunostaining (Fig. 3). In each case, the site of injection (nuclear or cytoplasmic) was established by supplementing the samples with rabbit IgG. This particular protein was chosen because several groups have shown that it remains confined to the nucleus or the cytoplasm after injection into either (25, 43, 104); this is presumably due to IgG's lack of either an NES or an NLS and to the fact that its relative molecular mass (~160 kDa) is well in excess of the ~60-kDa diffusion limit of nuclear pores (15, 33, 90). All samples were analyzed by double-label immunofluorescence so that GST-containing proteins and rabbit IgG were visualized with TXRD and FITC, respectively. Importantly, the unit-length GST protein remained sequestered in the nucleus after its introduction there (Fig. 3b). This was not due to its being imported from the cytoplasm, since cytoplasmic injection, as judged by the localization of IgG, resulted in its continued presence there (data not shown). Therefore, GST appears to be an excellent substrate for testing sequences for NES activity, as it contains neither an NLS nor an NES.

Inspection of cells that had received nuclear injections of GST:hRev/WT revealed a striking difference between its distribution and that of coinjected IgG (Fig. 3c and d). Specifically, the fusion protein containing the wild-type hRev activation domain entered the cytoplasm, whereas IgG remained in the nucleus. In fact, additional time course experiments revealed that the nuclear export of GST:hRev/WT was complete by 5 min postinjection at 37°C (data not shown). In contrast to this result, proteins that harbored the nonfunctional activation domains of M10 or M29 failed to exit the nucleus and therefore exhibited the same staining pattern as that of GST alone (compare Fig. 3f and h with b). Importantly, identical results were obtained when the Rev-specific, as opposed to the GST-specific, antibody was used for detection (data not shown). This indicates that these fusion proteins maintained their integrities during the course of these experiments. In summary, these data clearly demonstrate that a functional HIV-1 Rev activation domain serves as an NES and that inactivation of this region with respect to biological activity correlates with the inhibition of export activity.

To confirm that the activation domain of HIV-1 Rev is a transferable NES, peptides of 15 amino acids that contained residues 74 to 84 of wild-type hRev and the M10 mutant were synthesized and covalently cross-linked via their amino-terminal cysteines to BSA. Subsequent electrophoretic analysis of BSA-hRev and BSA-M10 conjugates showed that an average of approximately 10 peptides had been appended to each BSA monomer (data not shown). These complexes were biotinylated in vitro, injected into the nucleus, and assessed for subcellular localization after incubation at 37°C for 30 min (Fig. 4). As expected and consistent with our findings for GST:hRev fusions, BSA-hRev was efficiently exported to the cytoplasm (Fig. 4a), whereas BSA-M10 remained confined to the nucleus (Fig. 4b).

NES-mediated nuclear export is temperature dependent. Signal-mediated transport of macromolecules across the nuclear membrane has been shown to be temperature sensitive and energy requiring (14, 17, 72, 84, 88). Indeed, we have previously demonstrated that nuclear export of the full-length HIV-1 Rev protein is temperature dependent (77). To determine whether Rev activation domain-mediated transport in

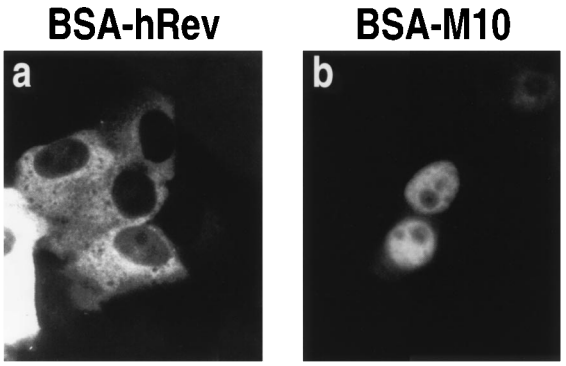

FIG. 4. A synthetic peptide of 15 amino acids derived from the HIV-1 activation domain acts as an NES. HeLa cells were injected into the nucleus with mixtures of rabbit IgG and biotinylated BSA-hRev (a) or BSA-M10 (b) conjugates and incubated for 30 min at 37°C. Samples were processed as described in the legend to Fig. 3 by using an FITC-conjugated donkey anti-rabbit antibody to detect IgG and TXRD-conjugated streptavidin to detect BSA conjugates; only the localization patterns of BSA conjugates are shown.

the absence of RNA binding is also energy dependent, we assessed the effects of temperature on nuclear export of GST:hRev/WT (Fig. 5). Cell monolayers were chilled on ice for 30 min prior to nuclear injection with GST:hRev/WT and then either warmed to 37°C for 15 min (Fig. 5a) or maintained on ice for a further 15 min (Fig. 5b). In contrast to its expected translocation to the cytoplasm at 37°C, the GST:hRev/WT protein was clearly retained in the nucleus at 0°C. The subsequent warming of a parallel sample to 37°C revealed that this fusion protein could rapidly enter the cytoplasm and was therefore still fully competent for export (data not shown). This observation indicates that nuclear export mediated by the NES of the HIV-1 Rev *trans* activator, like that of many RNAs and proteins, is fully dependent on energy utilization.

Identification of NESs in the Rev proteins of visna virus and EIAV. As discussed earlier, the possession of a Rev *trans* activator is not a unique property of HIV-1. In particular, all lentiviruses examined to date encode a Rev-like protein that acts to induce the export of unspliced viral mRNA to the cytoplasm. Further analysis of a number of these Rev proteins, namely, those derived from primate viruses HIV-2 (38) and simian immunodeficiency virus from macaques (21) as well as nonprimate viruses EIAV (35, 71), visna virus (102), and feline

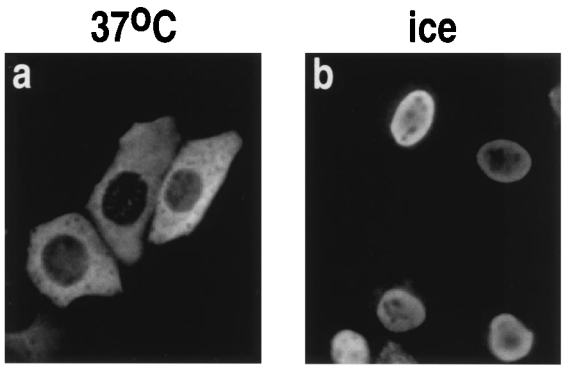

FIG. 5. Temperature dependence of Rev activation domain-mediated nuclear export. HeLa cells were chilled on ice for 15 to 30 min, injected with GST:hRev/WT supplemented with rabbit IgG, and either warmed to 37°C for 15 min (a) or held on ice for a further 15 min (b). Samples were processed as described in the legend to Fig. 3. Only the staining for GST:hRev/WT is shown.

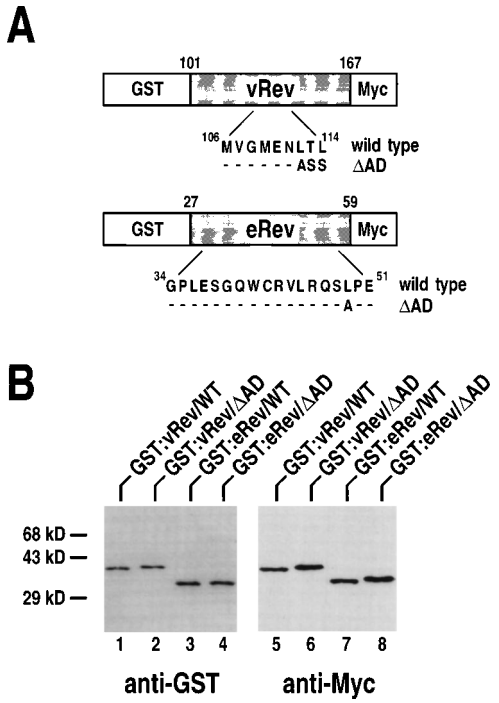

FIG. 6. Structure and purification of GST:vRev and GST:eRev fusion proteins. (A) The activation domains of wild-type and nonfunctional (ΔAD) visna virus (residues 101 to 167) and EIAV (residues 27 to 59) Rev proteins were fused in frame between the carboxy terminus of GST and the Myc epitope tag. The wild-type activation domain sequences as well as the residues altered in mutants are shown, as described in the legend to Fig. 2. (B) Western analysis of affinity-purified GST:vRev/WT (lanes 1 and 5), GST:vRev/ΔAD (lanes 2 and 6), GST:eRev/WT (lanes 3 and 7), and GST:eRev/ΔAD (lanes 4 and 8) fusion proteins. Filters were initially hybridized with murine monoclonal antibodies specific for GST (lanes 1 to 4) or the Myc epitope (lanes 5 to 8). The positions of prestained protein molecular mass (in kilodaltons [kD]) standards are indicated on the left.

immunodeficiency virus (71), has revealed that all of them have an activation domain. In each case, mutational inactivation of this region has been shown to inhibit RNA export and, when tested, to do so without impacting upon RNA binding. On the basis of our results for HIV-1 Rev, we predicted that all Rev activation domains would exhibit NES activity. To test this hypothesis, regions containing wild-type and nonfunctional (ΔAD) activation domains from the vRev and eRev proteins were fused to the carboxy terminus of GST (Fig. 6A) and purified by affinity chromatography. Western blotting analysis (Fig. 6B) using antibodies specific for GST (lanes 1 to 4) or for the Myc epitope tag at the carboxy terminus of each protein (lanes 5 to 8) confirmed that all four fusion proteins were intact and of the expected relative molecular mass (~34 kDa for GST:vRev and ~30 kDa for GST:eRev).

These four proteins were injected into HeLa cell nuclei, and their abilities to migrate to the cytoplasm were determined by immunofluorescence (Fig. 7). As predicted, both fusions that contained wild-type activation domains, GST:vRev/WT (Fig. 7a) and GST:eRev/WT (Fig. 7c), were efficiently exported from the nucleus. In contrast, the transport of proteins that harbored mutated activation domains, GST:vRev/ΔAD (Fig. 7b) and GST:eRev/ΔAD (Fig. 7d) was severely debilitated. However, unlike what was observed with nonfunctional activation domains of hRev, these two proteins, and especially GST:vRev/ΔAD, were not absolutely retained in the nucleus, as some staining of the cytoplasm could be discerned. Nevertheless, these data do demonstrate that the activation domains of

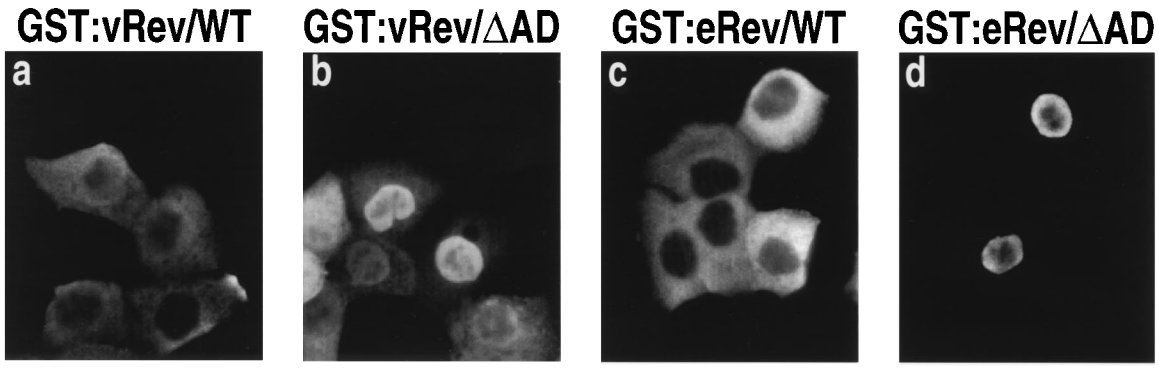

FIG. 7. The activation domains of the visna virus and EIAV Rev proteins are efficient NESs. HeLa cells were injected into the nucleus with mixtures of rabbit IgG and GST:vRev/WT (a), GST:vRev/ΔAD (b), GST:eRev/WT (c), or GST:eRev/ΔAD (d) and incubated for 5 min at 37°C. Samples were processed as described in the legend to Fig. 3, and the staining patterns for GST:Rev proteins are shown.

the visna virus and EIAV Rev proteins serve as NESs and that the functional inactivation of these sequences corresponds with a marked reduction of this activity.

Rev activation domain-mediated nuclear export is a saturable process. A defining feature of specific signal-mediated nuclear export is that it should be saturable as well as susceptible to inhibition by mutation (14, 57). Although we have shown that mutational disruption of the activation domain either prevents (hRev; Fig. 3 and 4) or severely reduces (vRev and eRev; Fig. 7) NES activity, the notion that protein sequence-determined nuclear export is truly specific would be further supported by a demonstration of saturability. To examine this issue with regard to the activation domain of HIV-1 Rev, we used BSA-hRev and BSA-M10 conjugates as wild-type and mutant competitors for the nuclear transport of GST:hRev/WT (Table 1). The use of BSA conjugates was considered particularly attractive since each carries approximately 10 cross-linked peptides; as a result, equal molarities of BSA conjugate and GST fusion equate to a 10-fold molar excess of competitor. Because we have already shown that BSA-hRev itself exits the nucleus (Fig. 4), an important consideration in the design of these experiments was to maximize the period during which BSA-hRev would be present in the nucleus and therefore available as a competitor. Given that we have already shown that activation domain-specific export is temperature dependent (Fig. 5), we therefore reduced the kinetics of export by performing these experiments at room temperature.

TABLE 1. NES-mediated export as a saturable process[a]

Test protein	Inhibition (%) of nuclear export by competitor[b]	
	BSA-hRev	BSA-M10
GST:hRev/WT	80 (49/61)	23 (26/111)
GST:vRev/WT	~90 (7/8)	35 (7/20)
GST:eRev/WT	80 (18/22)	30 (6/21)

[a] HeLa cells were injected into the nucleus with wild-type GST:Rev fusion proteins and either an ~10- (hRev) or ~20-fold (vRev and eRev) molar excess of hRev or M10 peptide cross-linked to BSA; all cocktails were further supplemented with rabbit IgG. Cells were incubated at room temperature for ~5 (vRev and eRev) or ~10 (hRev) min, and the localization of GST fusions was determined as described in the legend to Fig. 3.

[b] The inhibition of NES-mediated nuclear export was scored as the percentage of injected cells exhibiting nuclear localization of IgG that had retained a predominantly nuclear staining pattern for GST:Rev fusion proteins; the precise cell counts from a representative experiment are given in parentheses. The value for the GST:vRev/WT–BSA-hRev combination is shown as an approximation because of the relatively small number of nuclear injected cells in this sample in this particular experiment.

An examination of cells injected into the nucleus with BSA-hRev as the competitor revealed that activation domain-mediated export was clearly saturable since GST:hRev/WT was still predominantly nuclear in 80% of these cells at ~10 min postinjection. Importantly, by coinjecting the BSA-M10 mutant as a competitor, we were able to confirm that export inhibition is specific. In particular, GST:hRev/WT was cytoplasmic in the clear majority (77%) of cells examined 10 min after injection. In light of the currently popular view of Rev function, in which the activation domain is thought to interact with a cellular cofactor (protein) critical to *trans* activation, we therefore propose that BSA-hRev imparts its specific inhibition of nuclear export by binding to that factor and blocking its access to GST:hRev. During the course of these competition experiments, we noted that native BSA (i.e., BSA not conjugated to a peptide) displayed no detectable inhibition of GST:hRev export (data not shown). Thus, the mild inhibition observed with BSA-M10 could potentially be due either to weak interaction between the mutant peptide and the cofactor or to a nonspecific effect of having high concentrations of BSA-peptide conjugates in the nucleus.

On the basis of the shared attributes of lentivirus Rev activation domains (NES activity and activation of RNA export), it might be predicted that all of them would interact with the same host cell cofactor(s). As a test of this hypothesis, we used BSA-hRev and BSA-M10 conjugates as competitors for the nuclear export of the noncognate GST:vRev/WT and GST:eRev/WT fusion proteins (Table 1). Consistent with specific inhibition of HIV-1 Rev-mediated protein export, the BSA-hRev conjugate competed for the nuclear export of GST:vRev/WT and GST:eRev/WT with considerably more efficiency than did the BSA-M10 conjugate. We did find, however, that it required twice as much BSA-hRev to significantly inhibit the nuclear egress of the vRev and eRev activation domain-containing proteins than it did to inhibit the export of the corresponding HIV-1 Rev fusion protein. Nevertheless, the fact that the nuclear export of all three wild-type activation domains was clearly sensitive to sequence-specific inhibition by the wild-type BSA-hRev competitor implies that these domains interact with the same cellular cofactor(s).

DISCUSSION

In this study, we used the microinjection of somatic cells followed by immunostaining to demonstrate that the activation

domains of the HIV-1, visna virus, and EIAV Rev proteins are transferable NESs. In accordance with the criteria that have been established for other nuclear-cytoplasmic trafficking pathways, namely, NLS-dependent protein import, U small nuclear RNP import, and RNA export (14, 31, 40, 56, 57, 74), we provide three lines of evidence to suggest that NES-mediated export is both sequence specific and energy dependent. First, we have demonstrated the specificity of NES activity by showing that missense mutations within these sequences which abolish biological activity either block (hRev) or markedly impede (vRev and eRev) the transport of activation domain-containing fusion proteins to the cytoplasm (Fig. 3, 4, and 7). Second, as a further demonstration of specificity, we have shown that the export of each wild-type GST-activation domain fusion protein is saturable in that it was subject to specific repression by an excess of the wild-type HIV-1 Rev activation domain (Table 1). Third, we have demonstrated that nuclear export mediated by the HIV-1 Rev activation domain is temperature dependent and therefore an active process (Fig. 5).

We previously proposed that the translocation of Rev to the cytoplasm may itself be a key aspect of the process by which this protein activates viral mRNA export. One set of data that supported this model was derived from the analysis of activation domain mutants of HIV-1 Rev (77). Here, it was found that inhibition of biological activity always correlated with a loss of nuclear export potential. However, although these observations were consistent with the above-mentioned model for Rev function, it is also possible that Rev is carried to the cytoplasm as a consequence of being bound to RNA already destined for export. Even though this possibility may seem unlikely, especially in view of the fact that Rev exits the nucleus in the absence of its RNA ligand (the RRE) (59, 77, 87), it was essential to show that activation domain-dependent nuclear export could take place in the context of a protein which lacked RNA binding capability. To this end, we have therefore shown that activation domain-containing regions of HIV-1, visna virus, and EIAV Rev, which are themselves distinct from the sequences involved in RNA binding, can confer nuclear export on two different non-RNA-binding proteins. Once again the likely relevance of Rev export to the *trans* activation of RNA transport was further underscored by the finding that disruption of these activation domains profoundly inhibited NES activity.

The conclusion that Rev activation domains are autonomous and transferable NESs is therefore consistent with the idea that these sequences direct unspliced viral mRNA to the cytoplasm by providing export RNPs with the required targeting information. In agreement with this model, it has recently been shown by using microinjected *Xenopus* oocytes that RNA with an otherwise nonexportable adenosine cap at its 5′ terminus can be driven into the cytoplasm by interacting with the Rev protein of HIV-1 (30). Although this perhaps suggests that Rev supplies the only signal required for the transport of RRE-containing RNA to the cytoplasm, it is also possible that other components of export RNP, namely, other proteins or the RNA itself, provide additional signals that render viral RNPs maximally exportable in the context of virally infected cells.

It is interesting that the roles of the Rev *trans* activators in lentiviral mRNA export and that of the α subunit of karyopherin in protein import appear to be somewhat analogous. Specifically, not only do these proteins endow bound export or import substrates with the ability to travel through nuclear pores by an energy-dependent mechanism, but they themselves are able to traverse the nuclear membrane (40). The extent to which this analogy can be extended in terms of the involvement of other cellular factors remains to be determined. However, there are clearly some proteins, other than the components of the pores themselves, that are common to both pathways; in particular, the Ran GTPase (58, 75, 78), its associated partners (2, 9, 34), and the yeast protein Nop3p or Npl3p (32, 92, 106) have all been shown to be involved in the export of RNA from the nucleus and in protein import into the nucleus.

The cross-competition experiments discussed earlier (Table 1) suggest that the activation domains of the HIV-1, visna virus, and EIAV Rev proteins interact with the same host cell cofactor(s). Ultimate validation of this model will, of course, depend on the identification of the functionally relevant cofactor(s). Candidate cellular proteins that bind to the Rev protein of HIV-1 have already been identified by biochemical and genetic approaches (4, 24, 36, 64, 91, 100), and it is interesting that one of them, termed Rev/Rex activation domain-binding protein (Rab)/human Rev-interacting protein (hRIP), has been shown to interact with a variety of wild-type Rev activation domains, not with any of their nonfunctional mutant counterparts (4). In light of the absolute correlation between activation domain function, NES activity, and Rab/hRIP binding, it is therefore reasonable to propose that Rab/hRIP plays a key role in the nuclear export of Rev; indeed, we plan to evaluate this possibility in future studies. During our competition experiments, we also noted that export of the visna virus Rev activation domain and to a lesser extent that of the EIAV Rev activation domain were not inhibited as efficiently by BSA-hRev as was export of the cognate HIV-1 Rev activation domain. Although we do not yet understand the basis for these observations, we can formulate at least two hypotheses to explain them. First, the visna virus Rev and EIAV Rev activation domains may bind to the cofactor more efficiently than does the activation domain of HIV-1 Rev and, as a result, may be inhibited less effectively. Second and probably less likely, visna virus Rev and EIAV Rev may be able to access alternative export cofactors that can functionally substitute for the factor sequestered by BSA-hRev.

The question of how proteins exit the nucleus during interphase has been debated at some length in the literature (63). Although we and others have suggested that certain exported proteins likely contain NESs (72, 77, 104), it has also been proposed that nuclear export is determined not by specific peptide sequences but rather by the extent of nuclear retention (95). Therefore, the demonstration that lentivirus Rev proteins contain NESs proves that nuclear export can be governed by specific targeting signals. This does not exclude the possibility that export can also be determined by the totality of nuclear retention and can therefore occur in the absence of an NES. Indeed, we have argued previously that two such processes for protein nuclear export may exist. One characteristic that appears to distinguish these two pathways is the kinetics with which translocation to the cytoplasm takes place. In particular, the data presented suggest that NES-mediated export occurs far more rapidly than does NES-independent export (77, 95). In support of this hypothesis, it has recently been demonstrated that the heat-stable inhibitor (PKI) of cyclic AMP (cAMP)-dependent protein kinase, a protein that mediates rapid expulsion of the catalytic subunit of cAMP-dependent protein kinase from the nucleus, also harbors an NES (105). How widespread peptide NESs are remains to be determined; however, the fact that a number of proteins with diverse functions have been found to have nuclear export capabilities does suggest that many proteins harbor such sequences. It is therefore possible that NESs are involved not only in the trafficking of macromolecules from the nucleus but also in the transduction of intracellular signals between the nucleus and the cytoplasm.

Subsequent to the completion of this work, two other groups have also reported that the activation domain of HIV-1 Rev is an NES (29, 105).

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