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Nuclear Export of the Human Immunodeficiency Virus Type 1 Nucleocytoplasmic Shuttle Protein Rev Is Mediated by Its Activation Domain and Is Blocked by Transdominant Negative Mutants

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The human immunodeficiency virus type 1 nucleocytoplasmic shuttle protein Rev moves repeatedly between the cytoplasm, a perinuclear zone, the nucleoli, and nucleoplasmic speckles. In this study, we demonstrated by both indirect immunofluorescence and Western immunoblot analysis that nuclear exit of Rev transdominant negative mutants was defective compared with that of wild-type Rev. The basic and activation domains of Rev signal import and export, respectively, of Rev across the nuclear membrane. In cotransfection experiments, mutants containing mutations of Rev inhibited the nuclear egress of wild-type Rev, thus revealing a novel transdominant negative phenotype.

The expression of unspliced and incompletely spliced human immunodeficiency virus type 1 (HIV-1) mRNAs, which contain the information for the structural HIV components, is critically dependent on Rev at an unknown step(s) between transcription and translation (13, 30, 33). The Rev-dependent mRNAs are characterized by two types of *cis*-acting sequences, a single Rev-responsive element (RRE) partly overlapping *env* coding sequences (16, 17, 55), and several *cis*-acting repressive sequences (11, 19, 52, 70, 75). These sequences are removed in the completely spliced HIV mRNAs, which consequently do not require Rev for cytoplasmic appearance and translation. A current hypothesis is that Rev binds to the RRE and relieves nuclear retention of *cis*-acting repressive sequence-containing HIV mRNAs by unknown mechanisms (13, 33).

Site-directed mutagenesis of the 116-amino-acid Rev protein has delineated several functional domains. Mutations in a basic domain (amino acids 35 to 50) compromise nuclear and nucleolar accumulation and specific binding of Rev to the RRE RNA in vitro (5, 12, 35, 41, 53, 56, 63, 82). Mutations of amino acids flanking the basic domain (56, 63, 82) and in the carboxy-terminal part of Rev (15) may interfere with the propensity of Rev to multimerize. Amino acids necessary for Rev activity have been mapped to the amino-terminal part (37). The activation domain (amino acids 73 to 83), which contains characteristically spaced leucine residues, is necessary for Rev function in vivo. Mutations of critical amino acids in this domain generate transdominant negative mutants of Rev (Rev TD) (34, 35, 53, 56, 81). Acidic residues between amino acids 81 and 88 are also important (60). By definition, Rev TD inhibit the function of wild-type Rev (Rev wt) and outcompete Rev wt in a dose-dependent manner. As the molar ratio of Rev TD to Rev wt increases above 1, less and less Rev-dependent HIV proteins are made. It was shown that this effect was correlated to inhibition of Rev-dependent cytoplasmic accumulation of unspliced and singly spliced HIV mRNAs (53, 56, 80). The mechanism involved in regulation of HIV mRNA expression by the Rev activation domain is unknown. Indirect evidence suggests that the activation domain interacts with a host cell factor. In this paper, a precise function of the activation domain is reported; i.e., the domain is critically required for nuclear exit of Rev. In addition, the novel observation is made that Rev TD inhibit nuclear export of Rev wt in a dose-dependent manner. These observations elaborate further on the model of Rev as a nucleocytoplasmic shuttle protein which binds specifically to the mRNAs that it regulates and mediates the transport of the incompletely spliced RRE-containing mRNAs from the nucleus to the cytoplasm.

MATERIALS AND METHODS

Cell lines and plasmid constructs. The COS cell line, the transfection assays, the indirect immunofluorescence assay, and the plasmid pSVCMV*rev* expressing Rev wt have been described previously (39). The 293 cell line represents transformed human embryonal kidney cells obtained from the American Type Culture Collection (ATCC CRL 1573). Three different *rev* mutant plasmids, p*rev*81, M10, and p*rev*Δ18–23, collectively referred to as p*rev* TD, all with the simian virus 40 origin and the *rev* gene controlled by the cytomegalovirus immediate-early promoter, were used. In p*rev*81, the amino acid residue leucine 81 is changed to aspartic acid (80). In M10, the amino acid residues leucine 78 and glutamic acid 79 are substituted by aspartic acid and leucine, respectively (53). In p*rev*Δ18–23, the amino acid residues 76 to 84 are deleted (56). Plasmid pcDNA1E7 expresses both the Rev and the Env proteins and was a gift of J. Sodroski and R. Wyatt. Plasmid pSV1X1Δ1 expresses the Env proteins only when Rev is supplemented in *trans* and was a gift of M.-L. Hammarskjöld and D. Rekosh (32).

Antibodies. The anti-Rev monoclonal antibody (MAb) 1G10 binds to the C-terminal region of Rev, and the anti-Rev MAb 8E7 binds to the activation domain (39). MAb 8E7 recognizes Rev wt and Rev81 and M10 (but not RevΔ18–23) in the indirect immunofluorescence assay. In Western immunoblot analysis, MAb 8E7 bound poorly to Rev81. In the radioimmunoprecipitation assay, only Rev wt was precipitated by 8E7, confirming that the leucine-rich activation domain is an essential part of the epitope. For detection of Env gp160/gp120 in Western blot analysis, MAb ADP327 (79), provided by C. Thiriart and C. Bruck, Smith Kline Biologicals, Rixensart, Belgium, and H. C. Holmes, Medical Research Council, London, United Kingdom, was used. Polyclonal rabbit anti-gp160/gp120 antibody was obtained from American Biotechnology. The human anti-sm serum (Chemicon) recognizes the sm-antigen common to small nuclear ribonucleoprotein particles (snRNPs) in the nuclear speckles. MAbs 4F4 and 4B10 against heterogeneous nuclear RNP (hnRNP) C1/C2 proteins and hnRNP A1, respectively, were gifts of G. Dreyfuss and S. Piñol-Roma (66). The anti-mitochondrial MAb 1273 was from Chemicon.

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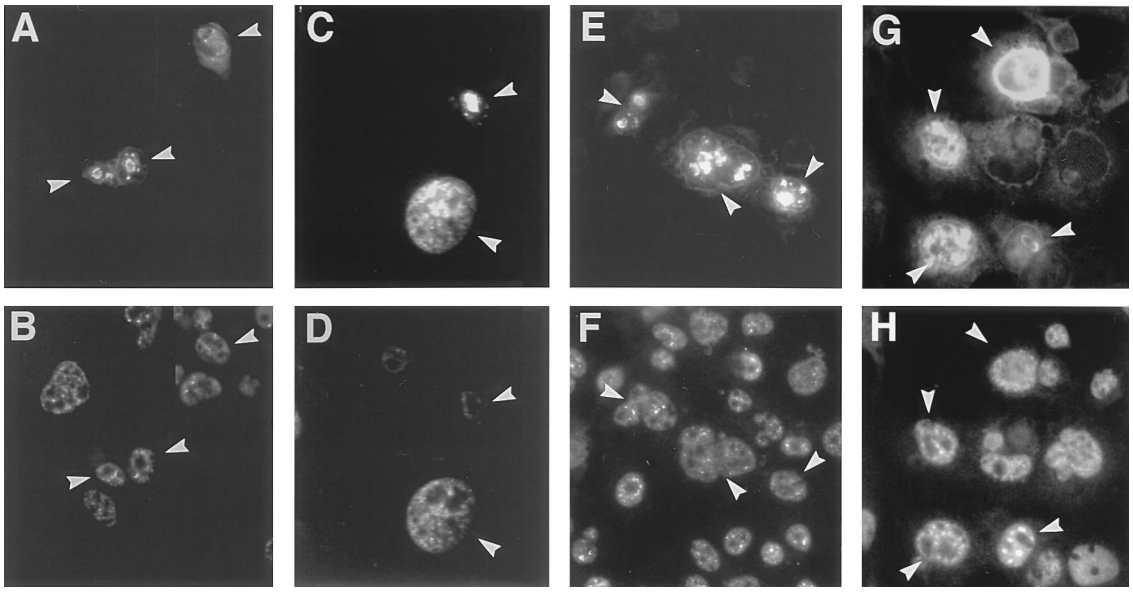

FIG. 1. Indirect immunofluorescent double labeling of COS cells transiently transfected with different Rev expressors and fixed in methanol after 48 h. (A and B) pSVCMV*rev*-transfected cells double labeled with anti-Rev MAb 8E7 (A) and human anti-sm serum (B). (C and D) p*rev*81-transfected cells labeled with 8E7 (C) and anti-sm serum (D). (E and F) M10-transfected cells labeled with 8E7 (E) and anti-sm serum (F). (G and H) p*rev*Δ18–23-transfected cells labeled with 1G10 (G) and anti-sm serum (H). FITC-labeled anti-mouse and rhodamine anti-human were used as secondary antibodies for all samples.

Transfection and cotransfection. COS-1 cells were seeded in 35-mm plates and transfected by the DEAE-dextran method with 10 μg of the different *rev* plasmids, 15 μg of pSV1X1Δ1, or 2 μg of pcDNA1E7 per plate. In the cotransfection experiments, the DNA concentration was held constant by the inclusion of pGEM7(Zf)+ (Promega Biotec). For Western blot analysis of nuclear and cytoplasmic extracts, COS-1 cells were plated in 100-mm plates and transfected with a total of 80 μg of plasmid by the lipofectamine procedure of Gibco BRL. 293 cells were plated in 60-mm plates and transfected with 5 μg of pSVCMV*rev* or the p*rev* TD by the lipofectamine procedure. After 24 h, the transfected cells were seeded onto 12-mm coverslips precoated with poly-L-lysine in 24-well plates. The next day, the cells were incubated with 10 μg of actinomycin D per ml and 50 μg of cycloheximide per ml for 3 h. Control coverslips were incubated in fresh medium without inhibitors. The coverslips were washed, fixed in ice cold methanol, and stored at −20°C.

Immunofluorescence analysis. The coverslips with methanol-fixed COS or 293 cells were washed twice with phosphate-buffered saline (PBS), blocked with 0.5% bovine serum albumin (BSA) in PBS for 15 min, and incubated for 1 h with the anti-Rev MAb 8E7 or 1G10. In the assay with COS cells, fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody diluted 1/100 (Boehringer, Mannheim, Germany) was used as the secondary antibody. Double labeling was performed with the rabbit anti-gp160/gp120 antiserum diluted 1/10 (American Biotechnology) or the human anti-sm serum (Chemicon) diluted 1/25, with Texas red-labeled anti-rabbit antibody diluted 1/100 (Amersham, Amersham, United Kingdom) or rhodamine-conjugated anti-human antibody diluted 1/50 (Boehringer), respectively. Different combinations of the primary and secondary antibodies were included to rule out nonspecific cross-reactions. For amplification of the signal in the 293 cells, the coverslips were incubated for 30 min. with biotin-labeled rabbit anti-mouse diluted 1/500 (Dakopatts, Glostrup, Denmark) and 30 min with FITC-conjugated streptavidin diluted 1/100 (Pierce, Rockford, Ill.). The coverslips were washed three times in PBS after each incubation. After a short rinse in distilled water, the coverslips were mounted in 7-μl SlowFade (Molecular Probes Inc.). The samples were viewed and photographed at a magnification of ×400 in a Nicon Microphot-SA with Epi-Fluorescence attachment connected to a Nicon FX-35DX camera. The micrographs were scanned with the Adobe Photoshop program and a Microtek Scanmaker IIx flatbed scanner.

Western blot analysis of nuclear and cytoplasmic fractions of cells. Transfected COS cells grown in 100-mm plates were trypsinized and pelleted in complete medium, resuspended in 1 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 2% polyvinylpyrrolidone [90 kDa], 0.2% BSA). Nonidet P-40 (NP-40) was added to a final concentration of 0.5%. The solution was shaken vigorously for 5 s, immediately underlaid with 24% sucrose in the same buffer except for NP-40, and centrifuged for 3 min at 1,500 × *g*. The sucrose and interface were discharged, and the remaining 700-μl cytoplasmic fraction was mixed with 175 μl of 4× sample buffer (0.2 M Tris-HCl [pH 6.8], 36% glycerol, 18% 2-β-mercaptoethanol, 40% sodium dodecyl sulfate, pyronin B), boiled for 3 min, and stored frozen until electrophoresis. The nuclear pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–1.5 mM $MgCl_2$–0.5% NP-40, 240 μl of 4× sample buffer was added, and the mixture was boiled and stored frozen. The samples were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide) and transferred to nitrocellulose (pore size, 0.2 μm; Schleicher & Schuell). The nitrocellulose membranes were blocked overnight in a solution containing 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 1% boric acid, 0.1% sodium hydroxide, 0.9% NP-40, 1.5% BSA, and 0.05% gelatin. Primary antibodies were added for 1 h. The ECL (enhanced chemiluminescence) system (Amersham) was used for detection as previously described (38). The films were scanned with the Adobe Photoshop program and a Microtek Scanmaker IIx flatbed scanner. Bio-Rad prestained SDS-PAGE low-molecular-weight standards were electrophoresed and transferred to membranes as molecular weight markers.

Western blot analysis of COS cells expressing Env proteins. Transfected COS cells grown in 35-mm plates were trypsinized and pelleted in complete medium, resuspended in 0.1 ml of 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–1.5 mM $MgCl_2$–0.5% NP-40 before addition of 50 μl of 4× sample buffer, boiling, and freezing. Lysates of Molt3IIIB cells chronically infected with the HIV-1 IIIB strain were used as control for antibody detection of gp160/gp120. The samples were separated by SDS-PAGE (7.5% polyacrylamide), electrophoretically transferred to nitrocellulose (pore size, 0.45 μm; Amersham), and analyzed by the ECL system. Both high- and low-molecular-weight prestained standards (Bio-Rad) were applied.

RESULTS

Subcellular localization of Rev TD proteins. The subcellular localization patterns of three different transdominant negative mutants of Rev, Rev81, M10, and RevΔ18–23 (Rev TD), were compared with Rev wt patterns (Fig. 1 to 3). Both Rev wt and the Rev TD localized to nucleoli and a perinuclear zone (Fig. 1 to 3) and colocalized with RNA-splicing factors in a speckled pattern in the nucleoplasm of methanol-fixed COS-1 cells (Fig. 1). No difference in the subcellular distribution of Rev wt expressed in COS cells (Fig. 2A) compared with 293 cells (Fig. 3A) was found. Likewise, no differences were found when Rev TD were expressed in COS cells (Fig. 2C, E, and G) and in 293 cells (Fig. 3C, E, and G). Previously, Rev wt was found to be distributed in similar patterns in transfected COS cells and HeLa cells and in HIV-1 IIIB-infected lymphoid CD4-positive C8166 cells, i.e., between the cytoplasm, a perinuclear zone, nucleoplasmic speckles, and the nucleoli (38, 39). Therefore, the subsequent experiments were done with COS cells. The most notable difference between Rev wt and Rev TD was the

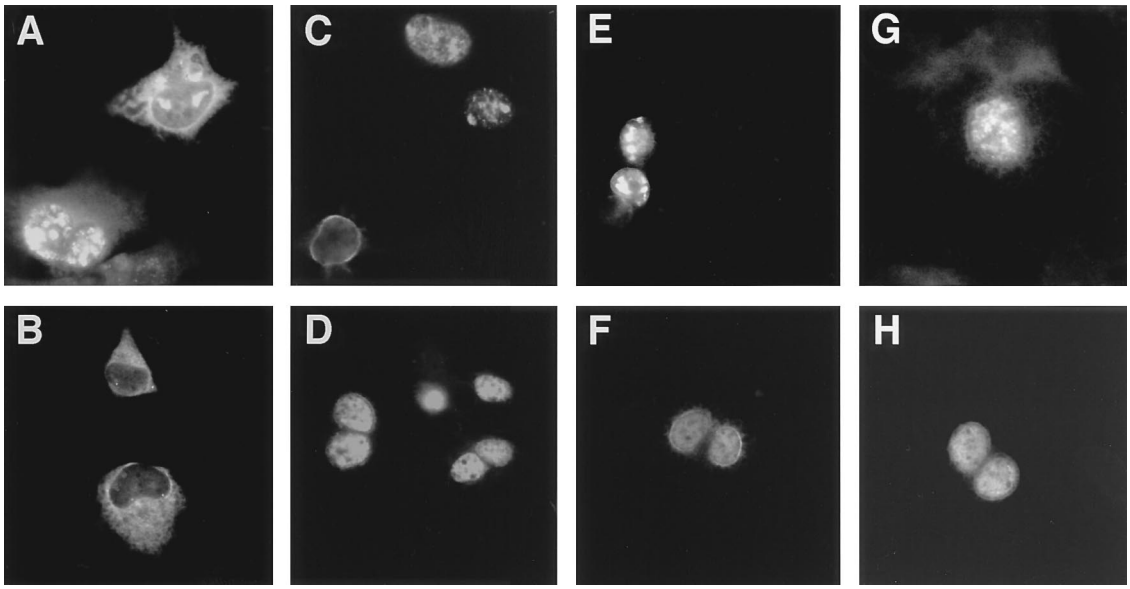

FIG. 2. Indirect immunofluorescence of COS cells transiently transfected with pSVCMV*rev* (A and B), p*rev*81 (C and D), M10 (E and F), and p*rev*Δ18–23 (G and H). Panels A, C, E, and G contain cells grown in normal medium. The parallel panels B, D, F, and H were treated for 3 h with 10 μg of actinomycin D per ml and 50 μg of cycloheximide per ml before fixation in methanol. The anti-Rev MAb 8E7 was used in panels A to F, and the anti-Rev MAb 1G10 was used in panels G and H. FITC-labeled anti-mouse antibody was used as the secondary antibody for all samples.

small amount of Rev in the cytoplasm of cells expressing Rev TD (Fig. 2C, E, and G and 3C, E, and G) compared with Rev wt (Fig. 2A and 3A). In addition, irregular nucleoplasmic accumulations of Rev TD were characteristically found in transfected cells (Fig. 1G and 2C [above], E, and G). A more pronounced accumulation of Rev TD in the perinuclear zone was typical in both COS and 293 cells (Fig. 1G, 2C, and 3E and G). The presence of short rays of Rev TD extending from the perinuclear zone toward the cytoplasm was another typical feature (Fig. 1G and 2C, E, and G).

Different response to actinomycin D treatment between Rev wt and Rev TD. As previously reported, Rev wt accumulated almost exclusively in the cytoplasm following 3 h of actinomycin D (10 μg/ml) and cycloheximide (50 μg/ml) treatment to inhibit RNA polymerase I and II and novel protein synthesis, respectively (38) (Fig. 2B and 3B). In contrast, the three Rev TD components (Rev81, M10, and RevΔ18–23) were still found almost entirely inside the nucleus except for the perinuclear zone and the cytoplasmic rays (Fig. 2D, F, and H and 3D, F, and H) following 3 h of actinomycin D and cycloheximide

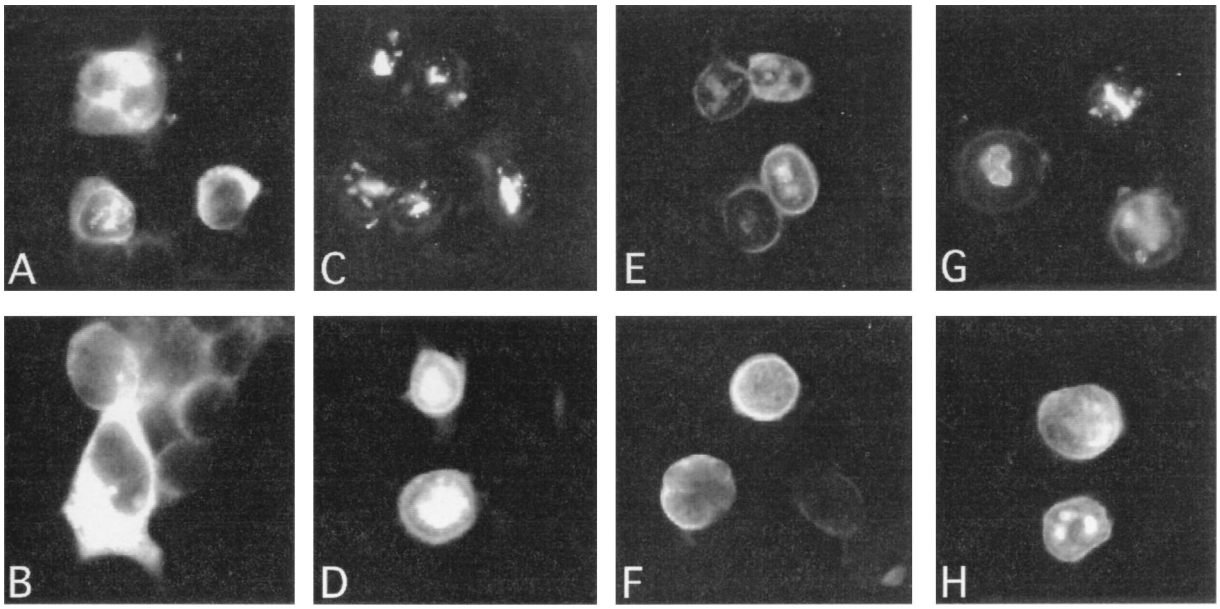

FIG. 3. Indirect immunofluorescence of 293 cells transiently transfected with Rev wt and Rev TD mutants, as in Fig. 2. (A, C, E, and G) cells grown in normal medium. (B, D, F, and H) Parallel experiments in which cells were treated for 3 h with 10 μg of actinomycin D per ml and 50 μg of cycloheximide per ml before fixation in methanol. A mixture of the anti-Rev MAbs 8E7 and 1G10 was used as the primary antibody followed by biotin-labeled anti-mouse conjugate and FITC-conjugated streptavidin.

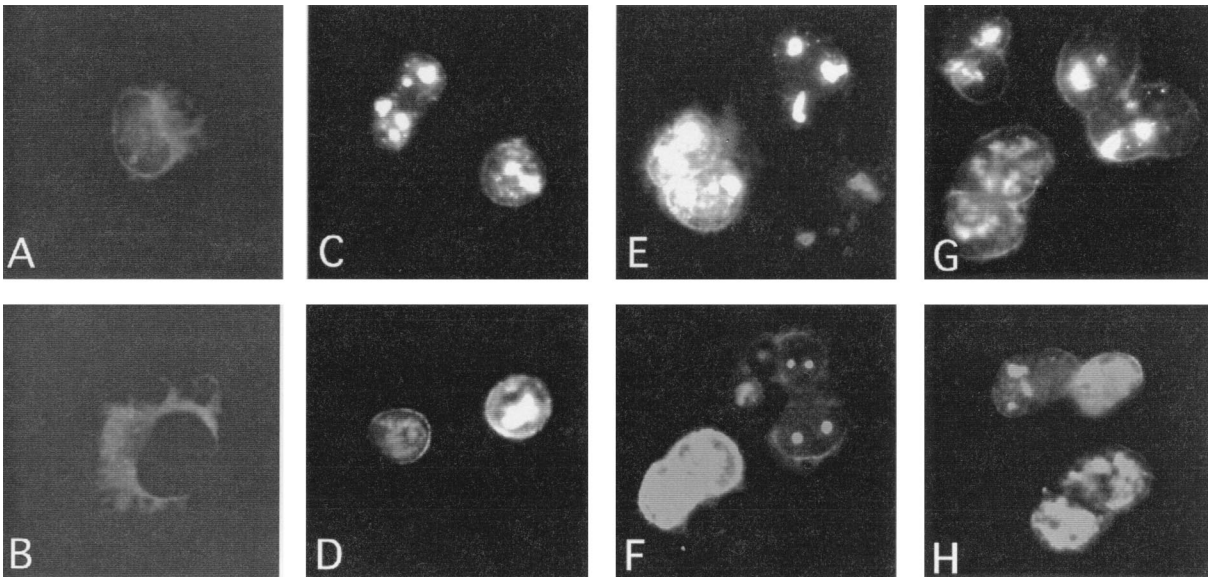

FIG. 4. Indirect immunofluorescence of COS cells transiently cotransfected with pSVCMV*rev* (A and B), p*rev*81 (C and D), M10 (E and F), or p*rev*Δ18–23 (G and H), together with the Rev-dependent RRE-containing Env expressor pSVSX1Δ1. The cells in panels B, D, F, and H were treated with 10 μg of actinomycin D per ml and 50 μg of cycloheximide per ml before fixation. Rev and Rev TD mutants Rev81 and M10 were detected with MAb 8E7, while the Rev TD mutant RevΔ18–23 was detected with MAb 1G10.

treatment. The Rev TD accumulated either in irregular patches in the nucleoplasm (Fig. 2D, F, and H and 3D and H) or in the perinuclear zone (Fig. 2D and F and 3D), often with fuzzy rays into the cytoplasm (Fig. 2F). In general, the effect of the actinomycin D treatment was to enhance the differences which already could be recognized between Rev wt and Rev TD in untreated cells.

Effect of RRE RNA on the subcellular distribution of Rev wt and Rev TD. Previously, we reported that Rev distribution and Rev nucleocytoplasmic shuttling were independent of the presence or the absence of the RRE or other HIV gene products (38, 39). Further experiments were done to assess the possible relative RRE-dependent effect on Rev distribution (Fig. 4). COS cells were cotransfected with Rev-expressing plasmids and the Rev-dependent RRE-containing Env expressor pSVSX1Δ1. No RRE-dependent change in the subcellular distribution of Rev could be detected. Rev wt (Fig. 4A) and Rev TD (Fig. 4C, E, and G) were distributed between the same compartments in the presence (Fig. 4) and absence (Fig. 1 to 3) of the RRE. Following 3 h of inhibition of transcription with actinomycin D, Rev wt accumulated in the cytoplasm (Fig. 4B), in contrast to Rev TD (Fig. 4D, F, and H), irrespective of the presence or the absence of the RRE RNA. The effect on subcytoplasmic Rev distribution dependent on target mRNA expression is under investigation. It is a formal possibility that Rev bound to the RRE is not detected by the anti-Rev MAbs used. This is unlikely, because three different anti-Rev MAbs (8E7, 1G10, and 9G2) (39) generated the same Rev distribution patterns in RRE-expressing cells as in cells not expressing RRE. As an additional control, an established RRE RNA gel mobility shift was used to examine Rev binding to the RRE in vitro (57). When different concentrations of each of the MAbs 8E7, 9G2, and 1G10 were included in the gel mobility shift, a supershift with four more slowly migrating bands resulted in nondenaturing acrylamide gel electrophoresis (results not shown). The supershift was specific for anti-Rev MAbs and did not occur in a negative control with corresponding concentrations of a MAb against a host cell component SC35 (results not shown). It was therefore concluded that the anti-Rev MAbs used detected Rev bound to the RRE in vitro.

Western blot analysis of Rev in nuclear and cytoplasmic fractions. To corroborate the effect of Rev TD on nucleocytoplasmic distribution of Rev, Western blot analysis of nuclear and cytoplasmic fractions of COS cell cultures transfected with Rev wt and the mutants was done. It was found that rapid leakage of proteins from the nuclei of fractionated cells represented a formidable methodological problem (1, 4, 18, 64). Therefore, controls for every fraction were performed with MAb 4F4 against the nuclear hnRNP C1/C2 proteins and MAb 4B10 against the nucleocytoplasmic shuttle protein hnRNP A1 (66). The hnRNP C1/C2 proteins are restricted to the nucleus in both actinomycin D-treated and untreated cells. The hnRNP A1 protein is confined mostly to the nucleus, but in cells incubated for 3 h with 10 μg of actinomycin D per ml, a large proportion of the hnRNP A1 exits to the cytoplasm (66). Figure 5A shows the Western blot analysis of nuclear and cytoplasmic fractions of COS cells expressing Rev wt and Rev81. The cells were incubated with actinomycin D and cycloheximide for 3 h prior to fractionation in order to enhance the difference between Rev wt and Rev81 and to avoid newly translated Rev in the experiment. Rev wt is visible in lanes 1 and 2, and Rev81 is visible in lanes 3, 5, and 7. It was found that the single-amino-acid substitution of Rev81 caused this mutant to migrate more slowly than Rev wt during electrophoresis. This difference in electrophoretic migration is in accord with previously published results (80). This single-amino-acid mutation within the epitope recognized by 8E7 apparently caused a weaker affinity between Rev81 and the MAb 8E7 than between Rev wt and 8E7. Therefore, three different, increasing concentrations of nuclear and cytoplasmic fractions of COS cells expressing Rev81 were loaded (lanes 3 to 8). The majority of Rev wt was found in the cytoplasm of actinomycin D-treated cells (lane 2), but a considerable amount of Rev was present in the nucleus as well (lane 1). In contrast, Rev81 was detected only in the nucleus (lanes 3, 5, and 7) and not in the cytoplasm (lanes 4, 6, and 8). Nuclear and cytoplasmic fractions of COS

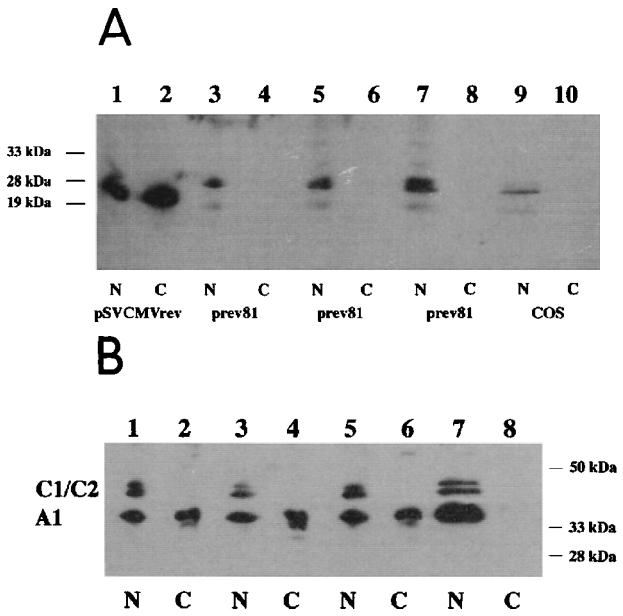

FIG. 5. ECL-Western blot analysis of nuclear (N) and cytoplasmic (C) fractions of transiently transfected COS cells. Actinomycin D (10 μg/ml) and cycloheximide (50 μg/ml) were used for 3 h before fractionation. Control COS cells (lanes 9 and 10) were not treated with actinomycin D and cycloheximide. Samples from the same experiment were used in panels A and B. Fractions corresponding to 4×10^5 cells were loaded into each well, except for panel A, lanes 5 to 8 (see below). (A) Lanes: 1 and 2, N and C fractions of COS cells transfected with pSVCMV*rev*; 3 to 8, fractions corresponding to 4×10^5, 8×10^5, and 12×10^5 cells transfected with p*rev*81; 9 and 10, fractions of nontransfected COS cells. MAb 8E7 was the primary antibody. (B) N and C fractions of transfected COS cells treated with actinomycin D and cycloheximide and the nontransfected COS cells probed with the anti-hnRNP C1/C2 MAb 4F4 and the anti-hnRNP A1 MAb 4B10. Lanes: 1 and 2, pSVCMV*rev*; 3 and 4, pSVCMV*rev* and p*rev*Δ18–23; 5 and 6, p*rev*81; lanes 7 and 8, nontransfected COS cells. The same nitrocellulose membrane was used with MAbs 4F4 and 4B10. The two films were superimposed after development.

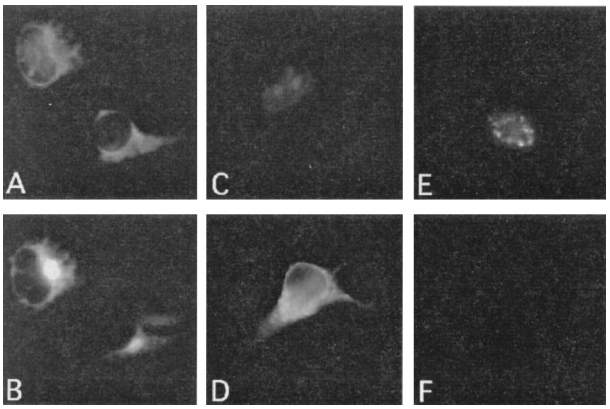

FIG. 6. Indirect immunofluorescent double labeling (A/B, C/D, and E/F, respectively) of COS cells transiently cotransfected with Rev-dependent Env expressors together with Rev wt expressor or the TD p*rev*Δ18–23. (A and B) pSVCMV*rev* and pSVSX1Δ1; (C and D) pcDNA1E7 encoding both the *rev* and the *env* genes; (E and F) pSVSX1Δ1 and p*rev*Δ18–23. Anti-Rev MAb 8E7 detected Rev wt (A and C). Anti-Rev MAb 1G10 detected RevΔ18–23 (E). Rabbit anti-gp120 serum was used in panels B, D, and F. FITC-labeled anti-mouse and Texas red-labeled anti-rabbit were used as secondary antibodies.

cells not exposed to actinomycin D or cycloheximide were used as controls. Weak nonspecific binding between MAb 8E7 and nontransfected COS cell nuclear antigens (lanes 9 and 10) could clearly be distinguished from the specific binding between MAb 8E7 and Rev. The controls for leakage of proteins from the nucleus to the cytoplasm during fractionation are shown in Fig. 5B. The same fractions which were analyzed for Rev wt (Fig. 5A, lanes 1 and 2) were analyzed for hnRNP C1/C2 and hnRNP A1 (Fig. 5B, lanes 1 and 2). Likewise, fractions were analyzed for Rev81 (Fig. 5A, lanes 3 to 8) and for hnRNP C1/C2 and A1 (Fig. 5B, lanes 5 and 6). The typical hnRNP C1 and C2 bands (41 and 43 kDa) were detected exclusively in the nuclear fractions of cells treated with actinomycin D (Fig. 5B, lanes 1, 3, and 5) and in the untreated COS cell controls (lane 7). In contrast, hnRNP A1 (36 kDa) was concentrated mainly in the nuclear fraction of cells not treated with actinomycin D (lanes 7 and 8) but responded to actinomycin D treatment with egress to the cytoplasm (lanes 2, 4, and 6). This demonstrated that no significant leakage of nuclear proteins occurred during subcellular fractionation and that the nucleocytoplasmic shuttle protein hnRNP A1 behaved as expected (66) in our system. Rev wt and hnRNP A1 thus responded similarly to actinomycin D treatment, in contrast to Rev TD, which were confined to the nuclear fractions independent of actinomycin D treatment. This difference between Rev wt and Rev TD is consistent with the parallel examination of the transfected cells by indirect immunofluorescence analysis (results not shown).

It must be emphasized that the subcellular fractionations were not routinely successful according to the criteria for hnRNP distribution. When the fractions were controlled for a mitochondrial marker antigen, significant contamination of the nuclear fraction was often found. This contamination was avoided by the use of a Dounce homogenizer with a type B piston, but intolerable nuclear leakage resulted (data not shown). The cytoplasmic (mitochondrial) contamination of the nuclear fractions in Fig. 5A and B was rather constant for each sample and was tolerable since it did not affect the relative differences between Rev wt, Rev TD, and hnRNP A1 distributions (data not shown). One conclusion is that the single-cell studies achieved a reliability and resolution unattainable by the analysis of cell fractions.

Immunofluorescence assays of Rev-dependent Env expression. The previously established effect of Rev TD is inhibition of the Rev wt-dependent cytoplasmic appearance of incompletely spliced HIV RRE mRNAs and the consequent shutdown of synthesis of HIV structural proteins (53, 56, 80). Two different cotransfection assays with COS cells were established to examine Rev-dependent Env expression. Double labeling was performed with the primary antibodies anti-Rev MAb 8E7 and the rabbit anti-gp160/120. In one assay, cotransfection with pSVCMV*rev* and the Env expressor pSVSX1Δ1 was required (double labeling in Fig. 6A and B). One cell with nucleolar, nucleoplasmic, and cytoplasmic Rev and one with most Rev in the cytoplasm are shown (Fig. 6A). Rev and Env often colocalized in the cytoplasm, with the conspicuous exception of Env associated with the Golgi complex (Fig. 6B). In the other assay, COS cells were transfected with pcDNA1E7, encoding both *rev* and *env* (double labeled in Fig. 6C and D). The level of Rev expression in pcDNA1E7-transfected cells varied but was always detectable in cells which also stained for gp160/120. Figure 6E and F shows that no Env was produced by pSVSX1Δ1 cotransfected with p*rev*Δ18–23.

The transdominant negative mutants of Rev inhibit nuclear export of Rev wt. The Rev TD expressed from p*rev*Δ18–23 are not recognized by MAb 8E7, since most of the epitope is deleted from this mutant. This made specific detection of Rev wt possible in cells coexpressing Rev wt and RevΔ18–23. Remarkably, cotransfection of COS cells with pSVCMV*rev* and p*rev*Δ18–23 caused the Rev wt to take on the Rev TD pheno-

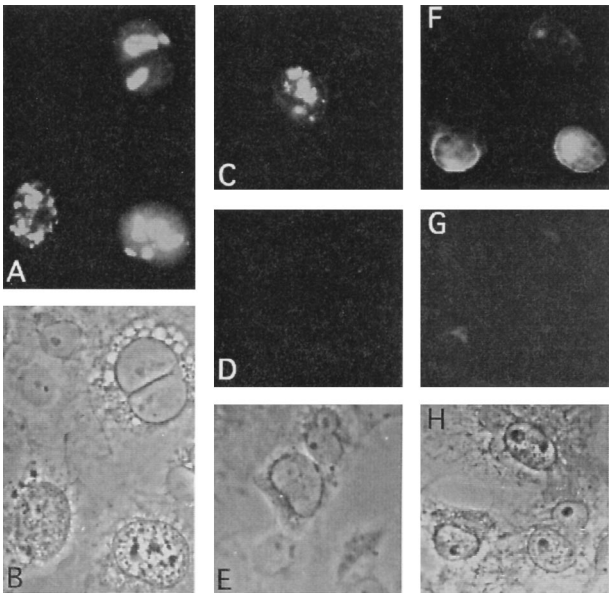

FIG. 7. Indirect immunofluorescent double labeling (C/D and F/G) and corresponding fields of phase-contrast of COS cells transiently cotransfected with different Rev and Env expressors. (A and B) pSVCMV*rev* and p*rev*Δ18–23. (C to E) p*rev*Δ18–23 cotransfected with pSVCMV*rev* and pSVSX1Δ1. (F to H) p*rev*Δ18–23 and pcDNA1E7. Double labeling of Rev wt and Env with the anti-Rev MAb 8E7 (A, C, and F) and rabbit anti-gp120 serum (D and G) is shown. FITC anti-mouse and Texas-red conjugated anti-rabbit secondary antibodies were used for all samples.

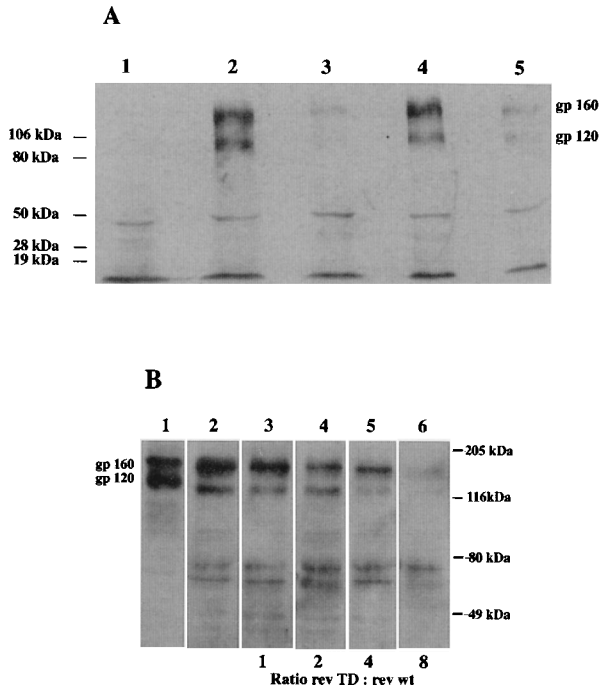

FIG. 8. ECL-Western blot analysis of total COS cell lysates demonstrating inhibition of Env expression by p*rev*Δ18–23. (A) Lanes: 1, lysate of cells transfected with pSVSX1Δ1 only; 2, pSVSX1Δ1 and pSVCMV*rev*; 3, pSVSX1Δ1 and pSVCMV*rev* and p*rev*Δ18–23 (equal amounts of pSVCMV*rev* and p*rev*Δ18–23 were used); 4, pcDNA1E7; 5, pcDNA1E7 and p*rev*Δ18–23 (eight times more p*rev*Δ18–23 [molar amount] than pcDNA1E7 was used). (B) Dose-response inhibition of Env expression from pcDNA1E7 by p*rev*Δ18–23 detected in total lysates of transfected COS cells. Lanes: 1, lysate of Molt3IIIB cells (no plasmid); 2, pcDNA1E7 only; 3 to 6, pcDNA1E7 and increasing amounts of p*rev*Δ18–23. The ratios of p*rev*Δ18–23 to pcDNA1E7 are indicated below lanes 3 to 6. The Env gp160/gp120 were detected with the anti-gp160/gp120 MAb ADP327.

type, with accumulation of Rev wt in the nuclei (Fig. 7A). The phase-contrast micrograph of the corresponding cells demonstrates nucleolar deformation (Fig. 7B), another typical feature of cells expressing Rev TD. Figure 7C to E represents Rev wt detection, Env detection, and phase-contrast, respectively, of COS cells triply transfected with pSVSX1Δ1, pSVCMV*rev*, and p*rev*Δ18–23. Again, Rev wt took on a Rev TD phenotype (Fig. 7C). The cell in Fig. 7D is double labeled for Env expression and is representative, since very few cells expressed detectable Env in this system. Equimolar amounts of pSVCMV*rev* and p*rev*Δ18–23 were used in this experiment. When equimolar amounts of pcDNA1E7 and p*rev*Δ18–23 were used in the second assay, the inhibition of Env expression was not absolute (Fig. 7F to and H are corresponding fields of Rev wt detection, Env detection, and phase-contrast, respectively). Notably, at the single-cell level, the appearance of Rev wt in the cytoplasm was correlated with Env production (lower left cell in Fig. 7F and G, respectively). The cells in which the Rev wt displayed a clear Rev TD phenotype with nuclear restriction did not produce Env (the lower right cell in Fig. 7F and G, respectively). M10 and p*rev*81 apparently had the same effects on Rev wt nuclear retention and inhibition of Env expression as p*rev*Δ18–23 (not shown). None of the available anti-Rev MAbs could, however, distinguish between M10 and Rev81 and Rev wt in the immunofluorescence assay.

A Western blot analysis (Fig. 8A) confirmed that the expression systems worked as intended. The gp 160/120 bands were detected with MAb ADP327. No Env was detected in crude lysates of COS cells transfected with pSVSX1Δ1 alone (Fig. 8A, lane 1), in strong contrast to cells cotransfected with pSVCMV*rev* (lane 2). When equimolar amounts of pSVCMV*rev* and p*rev*Δ18–23 were cotransfected with pSVSX1Δ1, a marked reduction of Env expression ensued (lane 3), consistent with the parallel immunofluorescence assay (Fig. 7C and D). To achieve a similar degree of inhibition of pcDNA1E7 Env production, an eightfold molar excess of p*rev*Δ18–23 was required for cotransfection (Fig. 8A, lanes 4 and 5). In Fig. 8B, a closer examination of the dose-dependent transdominant negative effect on Env-expression in one typical experiment is shown. Parallel examination of Rev and Env expression by indirect immunofluorescence revealed a strict correlation between restriction of Rev wt to the nucleus and decreased production of Env (results not shown).

Actinomycin D treatment of cells expressing both Rev wt and RevΔ18–23. COS cells were cotransfected with pSVCMV*rev* and p*rev*Δ18–23 in equimolar amounts, incubated 48 h later with 10 μg of actinomycin D per ml and 50 μg of cycloheximide per ml for 3 h, and then subjected to fixation or fractionation. In the immunofluorescence assay, it was evident that the Rev wt detected by 8E7 took on the Rev TD phenotype and was not exported to the cytoplasm under such conditions. In a subpopulation of cells, though, a mixed pattern was observed with substantial amounts of Rev wt in the cytoplasm. To examine the overall relationships more closely, a parallel culture was harvested into nuclear and cytoplasmic fractions and examined by Western blot analysis (Fig. 9). Purified recombinant Rev was used as a marker in lane 1. The ratio of nuclear Rev wt to cytoplasmic Rev wt was reversed when no p*rev*Δ18–23 was cotransfected (Fig. 9, lanes 2 and 3) compared with the ratio when an equimolar amount of p*rev*Δ18–23 was cotransfected (lanes 4 and 5). The absence of confounding nuclear leakage of hnRNP C1/C2 and A1 in these fractions was ensured by inclusion of samples from the fractions in Fig. 5B, lanes 3 and 4. Transfections, actinomycin D treatments, and fractionations were done in parallel for the results shown in Fig. 5 and 9.

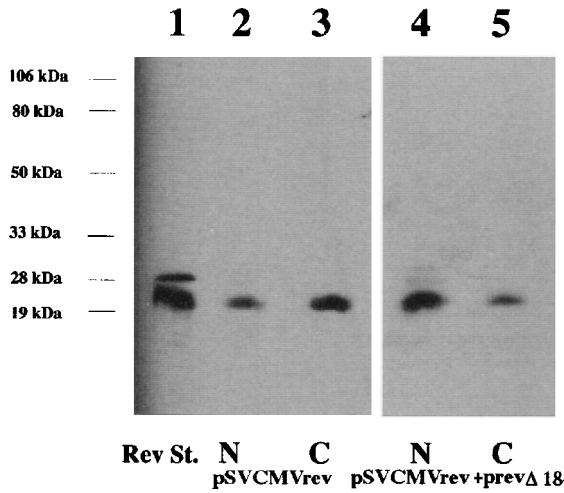

FIG. 9. ECL-Western blot analysis of nuclear (N) and cytoplasmic (C) fractions of transfected COS cells expressing Rev wt alone or Rev wt together with RevΔ18–23. Rev wt was detected with the anti-Rev MAb 8E7, which did not detect the Rev mutant encoded by plasmid p*rev*Δ18–23. Lanes: 1, 0.5 ng of purified recombinant Rev (ABT); 2 and 3, pSVCMV*rev*; 4 and 5, pSVCMV*rev* and p*rev*Δ18–23.

DISCUSSION

Rev is critical for the expression of the structural HIV-1 Gag/Pol and Env proteins (21, 44, 73, 76, 78). This positive regulation is exerted posttranscriptionally by increasing the cytoplasmic expression of unspliced and singly spliced HIV mRNAs from which the structural components are made. Many studies have found that Rev activity is associated with the export of the Rev-dependent mRNAs from the nucleus to the cytoplasm (19, 22, 32, 55). Divergent studies have found that in some systems the cytoplasmic appearance of incompletely spliced HIV mRNAs is not predominantly Rev dependent (2, 14, 47). However, the Rev-dependent mRNAs were not translated in the absence of Rev in these studies, an effect attributed to inefficient polysomal loading in the absence of Rev (2, 14). Different experiments have indicated Rev regulation of the mRNA splicing (8, 42, 43, 49, 77), of the mRNA transport (19, 22, 24, 32, 55), of specific transport of RRE RNA directionally across a nuclear membrane preparation (10, 65), and of the stability of Rev-dependent mRNA inside the nucleus (22, 54) at the nuclear level. The nucleocytoplasmic shuttling of Rev may serve to reconcile the apparently discrepant observations and may directly reflect the Rev mechanism of function. First, the same three leucine domain mutants (Rev TD) which are defective in the established Rev function of HIV mRNA expression (53, 56, 80) also are deficient in their ability to exit the nucleus (Fig. 1 to 5). Next, these export-negative Rev TD inhibit the nuclear export of the Rev wt; i.e., they display a transdominant negative phenotype (Fig. 7). There is a parallel dose-response effect between the ability of Rev TD to inhibit the nuclear export of Rev wt and to inhibit Rev-dependent Env production (Fig. 7 and 8). The Rev TD inhibit nuclear export of Rev-dependent mRNAs (53, 56, 80) and are shown in the present work to transdominantly inhibit the nuclear exit of the Rev wt protein itself (Fig. 7 and 9). The definitive proof that the Rev nucleocytoplasmic shuttling reflects its mechanism of function will, however, require the demonstration of Rev-dependent release of HIV mRNAs from the splicing machinery in vivo followed by Rev and RRE RNA cotransport to the translational machinery.

Although the three Rev TD were defective in their ability to exit the nucleus, they were still imported into the nucleus and the nucleolus (Fig. 1 to 3, 6, and 7), and in the nucleoplasm they associated with RNA-processing factors in a speckled pattern like the Rev wt (Fig. 1). One arginine-serine-rich (RS) motif that confers speckled localization upon proteins has been identified (9, 48, 71). An RS motif cannot be recognized in Rev. However, it was recently reported that a protein which copurifies with alternative splicing factor SF2/ASF binds to the arginine-rich domain of Rev (50). SF2/ASF contains the RS motif (28, 45).

The difference in subcellular distribution between Rev wt and the three members of Rev TD is thus restricted to the export part of Rev nucleocytoplasmic shuttling. The woolly nucleoplasmic patches and disrupture of nucleolar structure in many cells expressing Rev TD may reflect aggregation due to blocked passage out of the nucleus (Fig. 1 to 4 and 7). The pronounced accumulation of the transdominant negative mutants in the perinuclear zone and in rays (fibrils) extending into the cytoplasm is more remarkable. It is surprising if a barrier to passive diffusion is located on the exterior of the nuclear membrane. It is possible that a critical step of active translocation of Rev is thus outlined. In one study involving reconstituted nuclear membranes, unidirectional Rev-dependent mRNA transport across the membrane was detected (65). Interaction between Rev and a nuclear scaffold nucleoside triphosphatase activity may be important at this step (10). Several morphological studies have noted the existence of fibrils extending from the nuclear pore into the cytoplasm (see, e.g., references 23, 26, 68, and 69). The molecular details of nuclear import start to be unraveled. A large number of proteins, including Rev, with nuclear localization sequences is known; a putative nuclear localization sequence receptor (29) and proteins required for the active translocation step of nuclear import have been identified (reviewed in references 27, 62, and 67). Many proteins, including Rev (38, 39, 61), travel repeatedly back and forth between the cytoplasm and the nucleus and are called nucleocytoplasmic shuttle proteins (3, 6, 31, 40, 51, 58, 59, 66). The import leg of the shuttling is presumably comparable to nuclear import as described above. In contrast, the details of the nuclear export await discovery. Several proteins which are actively and sequence-specifically transported into the nucleus seem to go back to the cytoplasm in a signal-independent way (31, 51, 74). This has led to the proposal (46) that nuclear exit is the default direction of protein flow if the protein is not specifically retained inside the nucleus by binding to nuclear structures (40). The leucine-rich activation domain of Rev is the first example of an amino acid motif which signals nuclear export; the same finding was reported independently following the submission of this manuscript (61). The nuclear export of Rev therefore represents an alternative, since it is signal dependent and active. An anti-Rev MAb (150 kDa) microinjected into the nucleus can be specifically carried to the cytoplasm by Rev (38). The normal cargo of the Rev protein may be the RRE RNA.

The transdominant negative phenotype of Rev TD, discovered in this study, provides new clues regarding the molecular mechanism of Rev function. Competition between Rev wt and Rev TD for binding sites on the RRE is not a sufficient explanation, since the Rev trafficking per se is not affected by the presence or the absence of the RRE RNA. A deficient interaction between Rev TD and a host cell factor would not lead to competition for a limited amount of a host cell export factor between Rev wt and the mutants. One possibility is that the host cell factor recognition sequence of Rev is composed of leucine domains from several oligomerized Rev proteins.

Leucine-rich repeat motifs, probably involved in protein-protein interactions, have been identified in a number of eukaryotic proteins. The leucine-rich repeat motif is different from the leucine zipper class of coiled coils (25). The leucine-rich domain of Rev may correspond to one unit of such a repeat motif, and oligomerization of Rev may be necessary to constitute a functional leucine-rich repeat. It has been shown that mutations of conserved hydrophobic amino acids within one of the five repeat units in the snRNP-A′ protein rendered the mutants inactive (25). The hypothesis that efficient interaction between Rev and a nuclear export protein depends on a composite leucine-rich motif made up by Rev oligomerization can explain the phenomena observed in the present study. Mixed multimers of Rev wt and transdominant negative mutants would be expected both to be deficient in nuclear export and to retain the Rev wt inside the nucleus. It is noteworthy that if mixed multimers are required for the observed Rev TD phenotype, the presence of the RRE cannot be critical for Rev oligomerization to occur in vivo (36, 57, 63) (Fig. 7A).

The present identification of a sequence specifically required for nuclear export of the nucleocytoplasmic shuttle protein Rev allows for a refined model of Rev activity. The basic domain has previously been shown to be necessary for nuclear and nucleolar import of Rev. We recently published the results that Rev colocalized with RNA splicing factors in the nucleoplasm of cells and that Rev was transported actively back into the cytoplasm (38, 39). Assuming that Rev acts as a molecular bridge between the RRE and a nuclear transport molecule, it is conceivable that Rev has the potential both to relieve its target RNA from the splicing machinery and to guide the RNA into the cytoplasm. The role of the activation domain of Rev would be to bind to the putative host cell nuclear export factor according to this model. Several Rev-binding molecules, one of which binds to the Rev activation domain (72), have recently been discovered (20, 50, 72). The discovery in Mason-Pfizer monkey virus of an RNA element which obviates the need for both the RRE and Rev when inserted into otherwise Rev-dependent mRNAs (7) supports the notion that Rev (or, rather, an oligomer of Rev) acts as a molecular bridge between the RRE and a host cell nuclear export molecule. The latter, as yet unidentified molecule(s) may directly or indirectly contact possible nuclear filaments along which host cell nucleocytoplasmic transport normally might occur.

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