

The Human Immunodeficiency Virus Type 1 Rev Protein Shuttles between the Cytoplasm and Nuclear Compartments

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A retroviral regulatory protein, Rev (regulator of virion protein expression), is made in cells infected by human immunodeficiency virus (HIV). Rev is essential for the completion of the retroviral life cycle and interacts with the host cell at some posttranscriptional step in order to express the incompletely spliced HIV mRNAs from which HIV structural proteins are translated. Neither the host cell components nor the mechanisms responsible for this important regulation have been defined. We now report that Rev is a nucleocytoplasmic shuttle protein which is continuously transported between the cytoplasm, the nucleoli, and nucleoplasmic speckles enriched in RNA splicing and processing factors. The results show that Rev has the potential to interfere specifically with the splicing of the HIV pre-mRNA in the nucleoplasm and, next, guide such mRNAs to the cytoplasm for translation.

The genetic information of human immunodeficiency virus (HIV), which causes AIDS, is contained in a 9-kb genome. Elaborate differential splicing of the full-length initial transcript places the various reading frames into position for subsequent translation, resulting in three size classes of HIV mRNAs (about 9, 4, and 2 kb) (20, 23, 41). The two larger-size classes of HIV mRNA, which are incompletely spliced, cannot be expressed as Gag/Pol and Env proteins, respectively, unless an HIV regulatory protein, Rev, is present (16, 35, 44, 47, 50). Rev itself and Tat (11), another essential HIV protein, are made from the doubly spliced mRNA species which are expressed independently of Rev. When HIV transcription is activated in the infected cell, the full-length mRNA is spliced two times by default and translated into Tat and Rev proteins. Tat boosts transcription from the HIV promoter by positive feedback so that larger amounts of Tat and Rev are made. When Rev accumulates above a threshold level in the cell, the unspliced mRNAs (*gag/pol*) and the singly spliced mRNAs (*env*) become preferentially expressed (38). The HIV life cycle thus consists of an early phase characterized by increasing Tat and Rev production from doubly spliced mRNAs and a late phase with favored expression of viral structural proteins from unspliced and singly spliced HIV mRNAs (28). The hallmark of the Rev-dependent HIV mRNAs is that they contain two types of *cis*-acting sequences, the Rev response element (RRE) and several *cis*-acting repressive sequences (42). The RRE is a sequence of 234 ribonucleotides present in the *env* region of the HIV mRNA between the two exons of Tat and Rev (13, 36). The RRE folds into a complex stem-and-loop structure *in vitro* and binds avidly and specifically to the basic domain of the Rev protein (reviewed in references 11 and 23). The *cis*-acting repressive sequences are less well characterized but are present both in the *gag/pol* region and in the *env* region (10, 32, 42, 46). A plausible hypothesis is that the Rev-RRE binding relieves the suppression caused by the *cis*-acting repressive sequences, possibly by displacing (nuclear) host cell

factors that bind to them. The host cell structures that Rev interacts with to accomplish its transregulation of the HIV life cycle have not been identified. Candidate host cell factors that interact with Rev have been found (15, 43). Apparently discrepant results have identified the host cell RNA splicing machinery, the mRNA transport system, or cytoplasmic events of mRNA expression as important targets for Rev regulation (summarized in references 27 and 33). In this study it is shown that Rev shuttles continuously between the cytoplasm and the nucleus. In the nucleus Rev associates with speckles enriched in mRNA processing factors and additionally accumulates in the nucleolus. The discrepant observations regarding the Rev mechanism of action can be understood in light of a new model in which the nucleocytoplasmic shuttle protein Rev directs Rev-dependent mRNAs away from the host cell splicing machinery and into the cytoplasm for translation and then returns to the nucleus for further rounds of HIV RRE-RNA export.

MATERIALS AND METHODS

Cell lines and virus. HeLa/*crev8* cells and HeLa/*cnef* cells (gifts from Bryan Cullen, Howard Hughes Medical Institute, Durham, N.C.) were made essentially as described for the HeLa/*ctat* cells (34). In the HeLa/*crev8* cells Rev is expressed from the full-length *rev* cDNA present in *pcrev* (35). HeLa/*crev8* cells were grown in Iscove's medium (Gibco) supplemented with 5% fetal calf serum and 5×10^{-7} M methotrexate. In order to inhibit RNA polymerase II transcription, 5 μ g of actinomycin D (Sigma) per ml was added; alternatively, 5,6-dichlororibofuranosyl benzimidazole (DRB) at a concentration of 100 μ M was used to inhibit transcription. Protein synthesis was blocked by the addition of 20 μ g of cycloheximide (Sigma) per ml. C8166 is a human T-lymphoblastoid cell line reported to carry but not to express human T-cell leukemia virus type I (45) and was grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

The HIV type 1 (HIV-1) IIIB strain was used for infection. When typical cytopathic effect appeared in the C8166 cells after 6 to 8 days, a small volume of the infected culture was inoculated into a healthy culture. Extensive syncytia then appeared within 36 to 48 h.

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Autoimmune sera and monoclonal antibodies. A Smith antigen (sm)-positive serum (Chemicon) generates a speckled pattern in the nucleoplasm of cells. Several mouse monoclonal antibodies (MAbs) doubly labeled the sm-positive speckles by indirect immunofluorescence of methanol-fixed cells. MAb Y12 (30) recognizes the sm antigen common to U1, U2, U4/U6, and U5 small nuclear ribonucleoproteins (snRNPs) which are concentrated in the speckles; MAb anti-B" (21) detects the U2 snRNP-specific B" protein in the speckles; MAb H20 (2) detects the specific modification of snRNAs by 2,2,7-trimethylguanosine (m₃G caps) present in the speckles. MAb anti-SC35 (18) detects an essential non-snRNP RNA splicing factor localized in nucleoplasmic speckles. SC35 colocalized only partially and variably with the sm antigen in our study. MAbs against heterogeneous nuclear (hn) RNPs A1 (MAb 4B10) and C (MAb 4F4) (9, 37) were used. Anti-human lamin A/C (31) was used as a negative control. Anti-C-terminal Rev polyclonal rabbit serum was a gift from M.-L. Hammar-skjöld and David Rekosh (University of Virginia, Charlottesville). The antimitochondrial MAb 1273 was obtained from Chemicon. A MAb against HIV reverse transcriptase has been described previously (49).

Generation and characterization of anti-Rev hybridomas. The generation of 14 anti-Rev mouse hybridomas has been described previously (27). The MAbs were mapped by using a panel of 14 overlapping synthetic peptides spanning the whole length of the Rev protein (American Biotechnology). 1G10 and 8E7 reacted specifically with synthetic peptides corresponding to Rev amino acids 96 to 105 (GVGSPQILVE) and 70 to 84 (PVPLQLPPLERLTLD), respectively. 8E7 belonged to the immunoglobulin G2a(κ) subtype, and 1G10 belonged to the immunoglobulin G2b(κ) subtype.

Immunofluorescence analysis. Coverslips with cells were washed twice in phosphate-buffered saline (PBS) and fixed in ice-cold methanol for at least 10 min. Following two washes in PBS, the coverslips were incubated in 0.5% bovine serum albumin (BSA) in PBS for 15 min. The first antibody at an appropriate dilution in PBS–0.5% BSA was added for 45 min. PBS washes were done prior to and following incubation with Texas red- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 30 min. Rhodamine anti-human (Boehringer), FITC anti-mouse (Boehringer), and Texas red anti-rabbit (Amersham) conjugates were diluted 1:100 in 0.5% BSA in PBS. The immunofluorescent staining patterns of nuclear antigens in paraformaldehyde-stained cells often differed from those in methanol-fixed cells. Several protocols including fixation of cells in 4% paraformaldehyde in PBS–0.05% Tween 20, followed by washes in PBS and blocking in 10 mM glycine, a wash in PBS followed by cold methanol, or immunofluorescence as above were compared. The latter protocol was tried with or without Tween 20 and, alternatively, by fixation in 4% paraformaldehyde–50% ethanol.

Cytoplasmic and nuclear fractionation of cells. HeLa/crev cells were grown to 70 to 80% confluency, trypsinized and pelleted in complete medium, and resuspended in a small volume of lysis buffer (10 mM Tris-Cl [pH 7.5], 150 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂, 2% polyvinylpyrrolidone [90 kDa], 0.2% BSA). Nonidet P-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 without Nonidet P-40, and pelleted for 3 min at 1,500 × g. The cytoplasmic fraction was mixed with loading buffer, boiled for 3 min, and stored frozen until electrophoresis. The nuclear pellet was resuspended in 10 mM Tris-Cl (pH 7.4)–10 mM NaCl–1.5 mM MgCl₂ before addition of loading buffer, boiling, and freezing.

Western blot (immunoblot) analysis. Standard 15% polyacrylamide gel electrophoresis was done. When the electrophoresis was completed, the separated proteins were transferred to polyvinylfluoride membranes by using a Bio-Rad transblotting unit. The Amersham enhanced chemiluminescence system (ECL) was used for detection according to the instructions except that the specificity was increased by dilution of the horseradish peroxidase secondary conjugate severalfold more than recommended. The substrate solution was diluted twice with distilled H₂O. ECL films were scanned by using the Adobe Photoshop program and a Mikrotek Scanmaker IIX flatbed scanner. Scanned data were analyzed by using the National Institutes of Health Image program, version 1.54, and the gel-plot macro.

Microinjection. HeLa/crev cells were grown in Iscove's medium supplemented with 5% fetal calf serum and 5 × 10⁻⁷M methotrexate on 12-mm-diameter Assistant glass coverslips. Eppendorf microcapillaries were loaded with 1 mg of anti-Rev MAb 1G10 and 1 mg of Texas red-conjugated dextran (500 kDa) per ml in 2 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.6)–150 mM KCl. An Eppendorf micromanipulator 5171 was connected to an Eppendorf microinjector 5242. Injection time was set at 0.3 s, and effective injection pressure varied between 10 and 30 hPa. The injection was done on the heat stage of a Zeiss microscope. Coverslips with cells were returned to incubation chambers at controlled temperature and CO₂ concentration immediately after injection. At the indicated times the coverslips were washed twice in PBS, fixed for 10 min in methanol at 4°C, washed in PBS again, blocked for 10 min in 0.5% BSA, and incubated with FITC anti-mouse antibody (Amersham) (dilution, 1:100) for 30 min.

RESULTS

Subcellular localization of Rev in a HeLa/crev cell line. An extensive characterization of Rev distribution using indirect immunofluorescence has been reported recently (27). Rev was found in the cytoplasm (Fig. 1C and G and 2A and C), in a perinuclear zone (Fig. 1A, C, and G and 2A and C), in the nucleoli (Fig. 1A, C, E, G, and I and 2A and C), and in a speckled pattern in the nucleoplasm (Fig. 1A, C, E, G, and I and 2A and C) of methanol-fixed cells. In the nucleoplasm Rev colocalized with the sm antigen, an epitope common to U1, U2, U4/U6, and U5 snRNPs, in a speckled pattern. The colocalization was shown with both an autoimmune sm-positive human serum and the anti-sm MAb Y12. Rev and sm antigen colocalization was demonstrated by using a rabbit polyclonal anti-Rev serum and Y12 (Fig. 1A and B, respectively). Rev was found to colocalize also with the RNA processing factors hnRNP C (Fig. 1C and D) and hnRNP A1 (Fig. 1E and F). Rev additionally colocalized with the snRNA component of the snRNPs in a speckled pattern (Fig. 1G and H), as determined by immunofluorescent double labeling between the rabbit anti-Rev serum and MAb H20, which binds to 2,2,7-trimethylguanosine caps of snRNAs. The specificity of the double labeling between Rev and the RNA processing factors in a speckled pattern in the nucleoplasm is underscored by the conspicuous labeling of Rev alone in other compartments of the same cells, i.e., the cytoplasm (Fig. 1C, G, and I), the perinuclear zone (Fig. 1A, C, E, G, and I), and the nucleoli (Fig. 1A, C, E, G, and I). In contrast, the Y12 (Fig. 1B), 4F4 (Fig. 1D), 4B10 (Fig. 1F), and H20 (Fig. 1H) MAbs usually displayed stronger diffuse nucleoplasmic staining than that for Rev in addition to the speckled pattern. The negative control showed the absence of any double labeling between anti-Rev and MAb antilamin (Fig. 1I and J, respectively). It must be

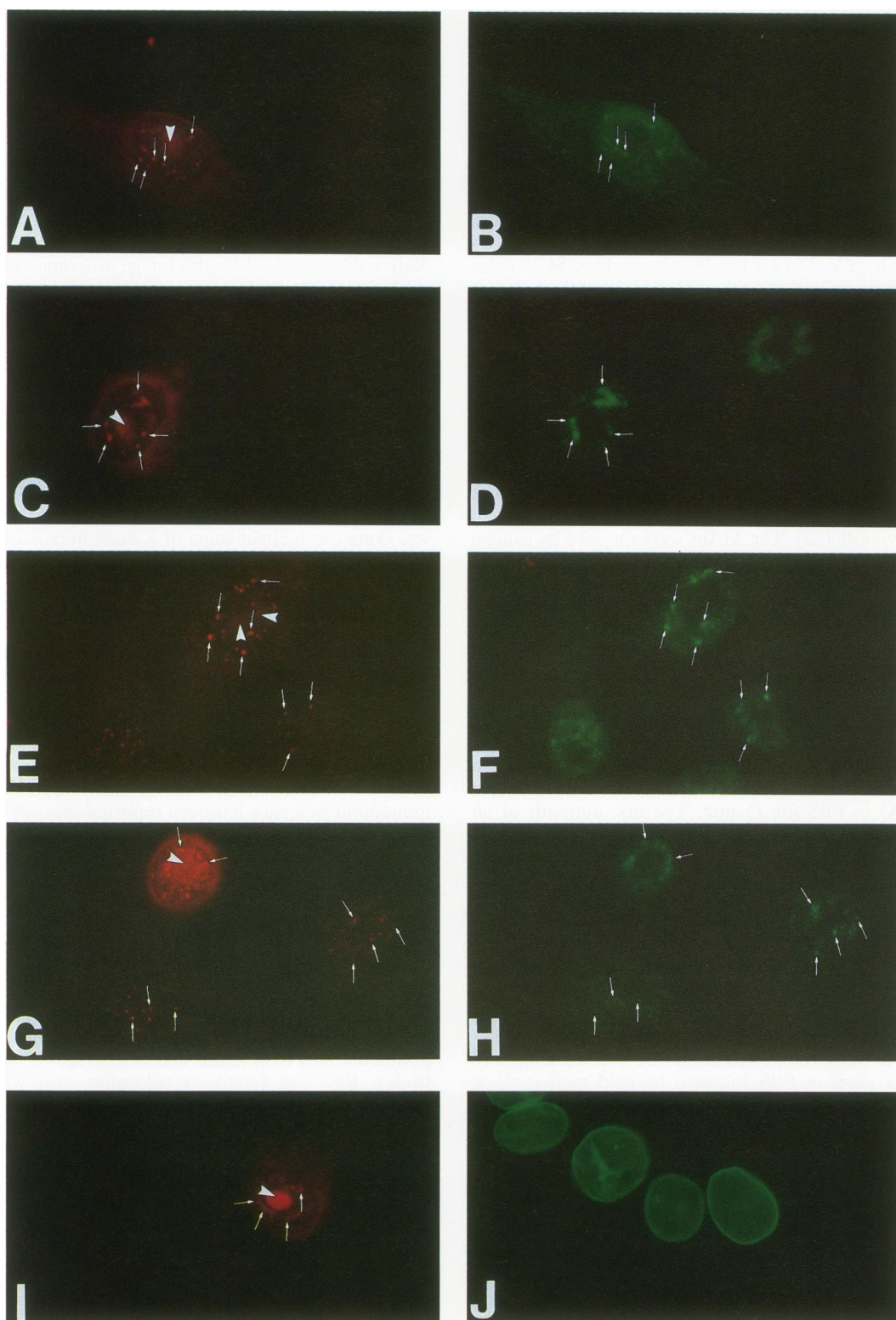


FIG. 1. Indirect immunofluorescent double labeling of HeLa/*crev* cells. (A, C, E, G, and I) Rabbit anti-Rev serum and a Texas red anti-rabbit secondary antibody (Amersham). (B) Y12 (an anti-sm MAb); (D) 4F4 (an anti-hnRNP C MAb); (F) 4B10 (an anti-hnRNP A1 MAb); (H) H20 (an anti-trimethylguanosine cap MAb); (J) antilamin MAb. The MABs were visualized with an FITC-conjugated anti-mouse antibody (Boehringer). Colocalization between Rev and factors associated with RNA processing is evident in nucleoplasmic speckles. The antilamin MAb provides a negative control. A pronounced cell-to-cell variation was characteristic for the abundance levels of Rev in contrast to the host cell proteins. Arrows, selected speckles; Arrowheads, nucleoli.

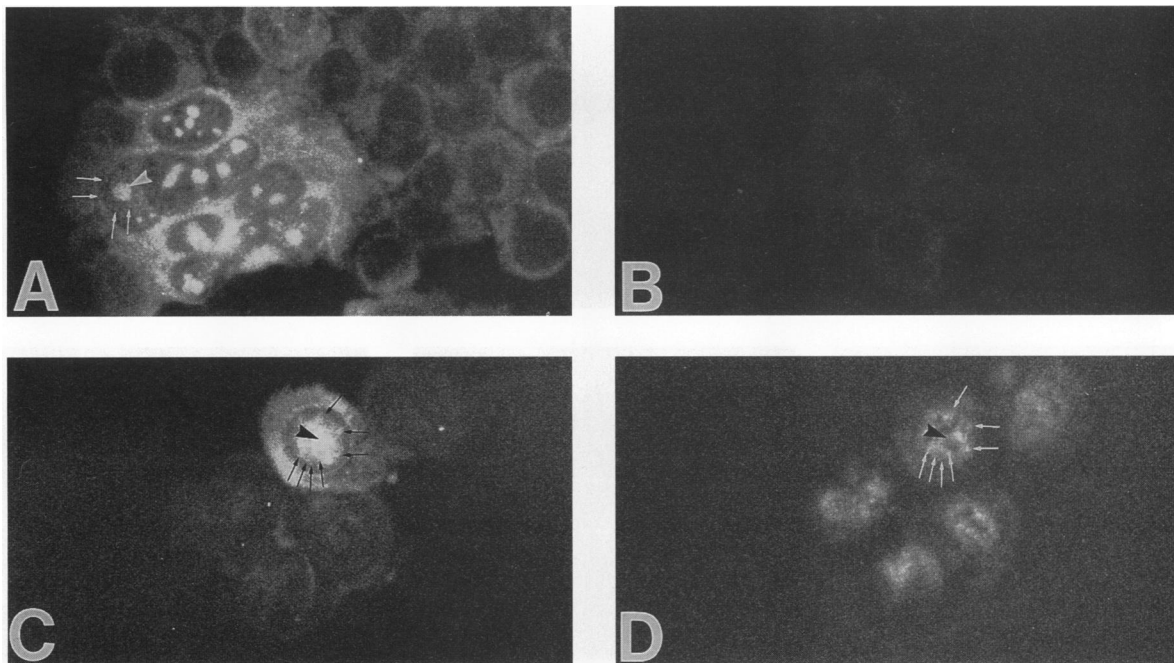


FIG. 2. C8166 lymphoid cells were infected with HIV-1 IIIIB for 48 h and deposited onto slides by using a cytospin centrifuge. The cells were fixed in methanol and examined by indirect immunofluorescence. (A) HIV-1 IIIIB-infected cells examined with 8E7, an anti-Rev MAb; (B) uninfected C8166 cells examined with 8E7; (C) HIV-1 IIIIB-infected cells examined with 8E7 (the FITC-conjugated anti-mouse immunoglobulin visualized bound 8E7); (D) same field as that in panel C labeled with human anti-sm-positive serum and rhodamine-conjugated anti-human antiserum. Selected speckles are marked by arrows. Arrowheads, nucleoli.

emphasized that the staining patterns of both Rev and the host cell nuclear factors were dependent upon the fixation method used. Attention has been directed to this problem previously (5, 6). In paraformaldehyde-fixed cells U1 snRNP-specific antibodies generated diffuse nucleoplasmic staining in contrast to both speckled and diffuse patterns of nucleoplasmic staining in methanol-fixed cells. Nucleoplasmic speckled double labeling between anti-m3G cap antibody and U1-specific antibody was visualized only in methanol-fixed cells and not in paraformaldehyde-fixed cells (5, 6). A similar situation was found in this study regarding the staining of hnRNPs A1 and C with MAb 4B10 and 4F4, respectively. In paraformaldehyde-fixed cells the staining with 4F4 and 4B10 was predominantly diffuse in the nucleoplasm regardless of permeabilization with Tween or methanol. In methanol-fixed cells nucleoplasmic hnRNP speckles were always evident in addition to variable diffuse staining. Paraformaldehyde fixation apparently masked nucleoplasmic Rev epitopes which were not successfully regenerated by several permeabilization protocols.

Rev subcellular distribution in HIV-1 IIIIB-infected lymphoid C8166 cells. Figures 2A and C represent the first in situ detection of Rev in infected cells. T-lymphoblastoid C8166 cells were infected by HIV-1 IIIIB and fixed in ice-cold methanol 48 h later. Although the strong Rev staining of the nucleolus dominated, colocalization between Rev and the sm antigen in a speckled pattern (Fig. 2C and D) was evident in doubly labeled cells. Rev was easily detectable in the cytoplasm and in the perinuclear zone (Fig. 2A and C). In uninfected C8166 cells, only faint nucleoplasmic background staining was visible with the anti-Rev MAb 8E7 (Fig. 2B).

Actinomycin D-induced and DRB-induced cytoplasmic accumulation of Rev in HeLa/crev cells. Since a substantial amount of Rev was found in the cytoplasm of both HeLa/crev

cells and HIV-1 IIIIB-infected lymphoid cells, the possibility that Rev is a nucleocytoplasmic shuttle protein was investigated. The first approach to the study of nucleocytoplasmic transport of Rev was based upon published work with the nuclear hnRNPs (37). When cells are grown in the presence of 5 μg of actinomycin D per ml to inhibit RNA polymerase II transcription, the nucleocytoplasmic shuttle protein hnRNP A1 accumulates in the cytoplasm. In contrast, hnRNP C proteins are restricted to the nucleus independently of transcription. These results were reproduced (Fig. 3) and extended by the parallel examination of the sm antigen and Rev. The sm antigen (Fig. 3A to C) and the hnRNP C proteins (Fig. 3J to L) remain in the nucleus when cells are exposed to actinomycin D. After 3 h of actinomycin D treatment Rev localization changed from predominantly nuclear to almost exclusively cytoplasmic (Fig. 3D to E). hnRNP A1 responded in a similar way to actinomycin D treatment, although usually with a significantly less pronounced exit from the nucleus compared with Rev (Fig. 3G and H and 3D and E, respectively). The export of Rev (and hnRNP A1) occurred at 37°C but not at 4°C, implying an active component of the transport process from the nucleus to the cytoplasm. The actinomycin D-induced cytoplasmic export of Rev (Fig. 3D and E) and hnRNP A1 (Fig. 3G and H) was partly reversible following a wash of the cells and incubation at 37°C in medium without inhibitors (Fig. 3F and I, respectively) or with cycloheximide only (data not shown). Rev accordingly has the potential to be transported bidirectionally over the nuclear membrane.

The absence or presence of cycloheximide at 20 $\mu\text{g}/\text{ml}$ during actinomycin D treatment at 5 $\mu\text{g}/\text{ml}$ did not affect the basic patterns of the actinomycin D-dependent changes of Rev subcellular localization. In a series of control experiments a different inhibitor of RNA polymerase II, DRB, was used

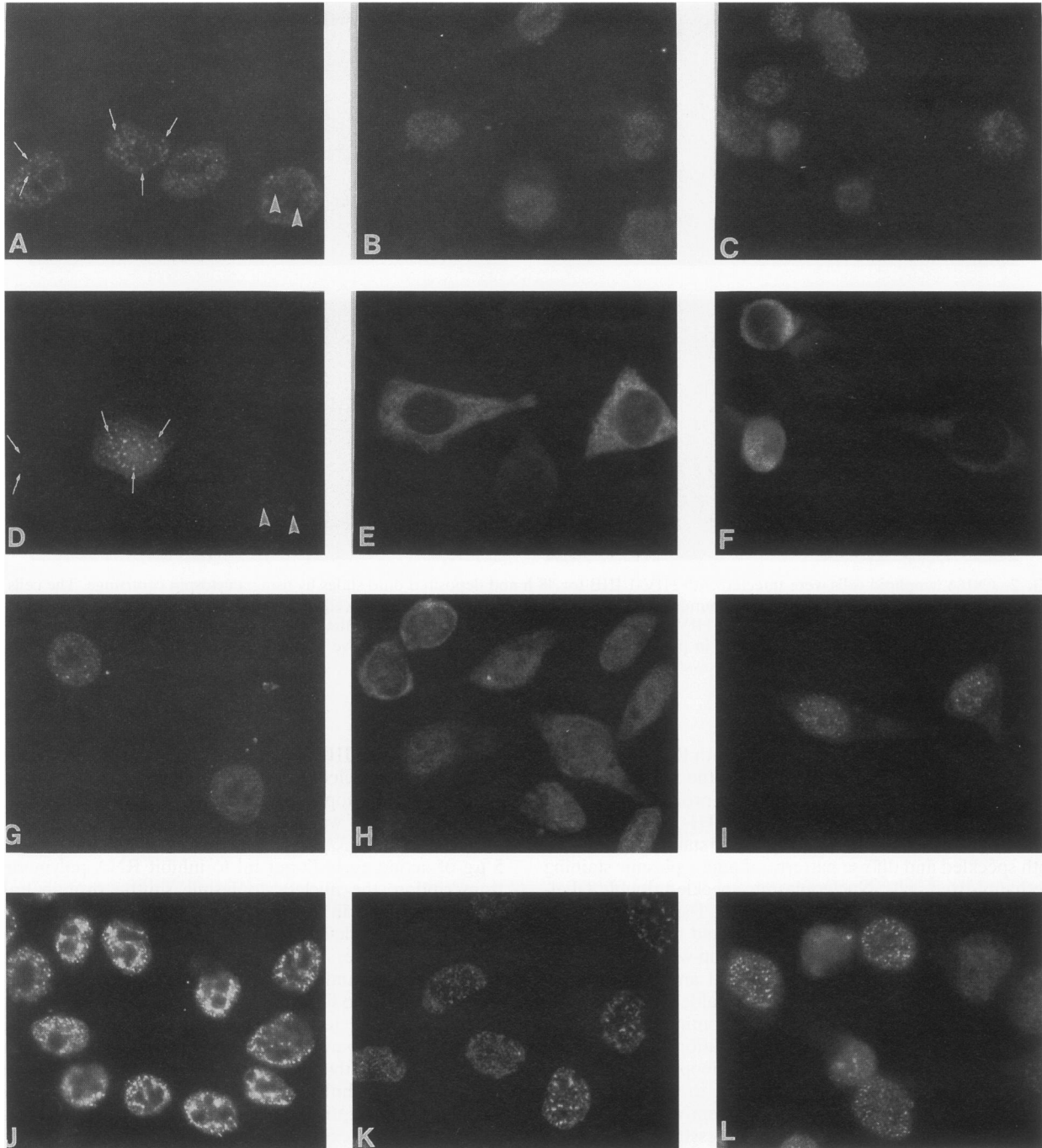


FIG. 3. HeLa/*crev* cells were incubated without (A, D, G, and J) or with (B, E, H, and K) 5 μ g of actinomycin D and 20 μ g of cycloheximide per ml for 3 h. (C, F, I, and L) Cells were incubated with actinomycin D and cycloheximide for 3 h, washed, and fixed after an additional 3 h in Iscove's medium with 5% fetal calf serum and without inhibitors of transcription or protein synthesis. (A to C) Human anti-sm-positive serum and rhodamine-conjugated anti-human secondary antibody; (D to F) 8E7 (an anti-Rev MAb) and FITC-conjugated anti-mouse antibody (double labeling of the samples in panels A to C, respectively); (G, H, and I) 4B10 (an anti-hnRNP A1 MAb) and FITC-conjugated anti-mouse antibody; (J, K, and L) 4F4 (an anti-hnRNP C MAb) and FITC-conjugated anti-mouse antibody; Arrows, selected doubly labeled speckles; arrowheads, nucleoli.

instead of actinomycin D. Very similar results were found. In the presence of 100 μ M DRB both Rev and hnRNP A1, but not hnRNP C, accumulated in the cytoplasm over 3 h. When the DRB-containing medium was replaced by DRB-free medium, nuclear reimport of Rev and hnRNP A1 was evident within 3 h (data not shown). The presence or absence of

cycloheximide during these experiments did not result in significant differences. Previously we found that incubation of cells with only cycloheximide at 20 μ g/ml did not change the apparent subcellular distribution of Rev for periods of up to 10 h or as long as the cells appeared viable (27).

In order to quantitate the relationships above, nuclear and

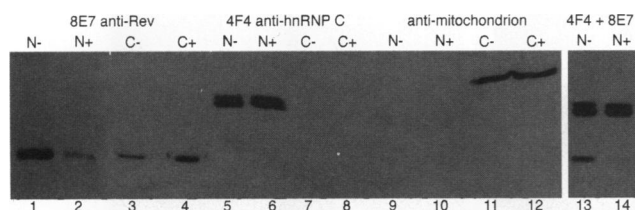


FIG. 4. Western blot analysis of nuclear (N) and cytoplasmic (C) fractions of HeLa/crev cells treated with actinomycin D (5 μ g/ml) and cycloheximide (20 μ g/ml) (+) and untreated controls (-). Membranes containing electrophoretically separated nuclear or cytoplasmic fractions were incubated with anti-Rev MAb 8E7 (lanes 1 to 4), anti-hnRNP C MAb 4F4 (lanes 5 to 8), or antimitochondrial MAb 1273 (Chemicon) (lanes 9 to 12). In lanes 13 and 14 double labeling using 8E7 and 4F4 shows the actinomycin D-induced decrease of the nuclear content of Rev compared with hnRNP C. A Macintosh Mikrotek scanner and the Adobe Photoshop program were used to assemble the data from two independent representative experiments (lanes 1 to 12 and lanes 13 and 14, respectively). Nucleus samples corresponding to 5×10^5 cells and cytoplasm samples corresponding to 10^5 cells (the cytoplasmic fraction was more diluted following fractionation) were loaded in the wells.

cytoplasmic fractions of HeLa/crev cells were analyzed by Western blot (Fig. 4, lanes 1 and 2 and lanes 3 and 4, respectively). As leakage from the nuclear to the cytoplasmic fractions proved a major problem, it was necessary to control each fractionation by using antibodies to nuclear (hnRNP C) (Fig. 4, lanes 5 to 8) and cytoplasmic (mitochondrion) (Fig. 4, lanes 9 to 12) marker antigens. The most important parameters for sufficient fractionation were to minimize the time between lysis and fractionation (less than 1 to 2 min) and to increase the colloid osmotic pressure in the lysis buffer by using 2% polyvinylpyrrolidone and 0.2% BSA (unpublished results). A selective decrease of Rev content in the nucleus following incubation for 3 h with actinomycin D and cycloheximide is evident in Fig. 4, lanes 1 and 2. A simultaneous increase of the Rev concentration in the cytoplasmic fraction of cells treated with inhibitors compared with untreated cells is shown in Fig. 4, lanes 3 and 4. The double labeling in Fig. 4, lanes 13 and 14, demonstrates the selective nuclear decrease of Rev compared with hnRNP C following inhibition of transcription and translation for 3 h. The Rev-specific and control bands of Western blot ECL films were subjected to densitometric scanning analysis. The nuclear fractions were normalized against the hnRNP C-specific bands before and after actinomycin D treatment. The Rev signal ratio in the nucleus before and after actinomycin D-plus-cycloheximide treatment averaged 8.3 in four independent experiments compared with a ratio of 0.8 for the hnRNP C bands. In the cytoplasm the ratio between Rev bands before and after actinomycin D-plus-cycloheximide treatment was about 0.3, while the corresponding mitochondrion marker band ratio was close to 1.

Active transport of Rev bidirectionally over the nuclear membrane following microinjection. This assay is based upon the assumption that a microinjected antibody can bind to its corresponding antigen *in vivo* and be carried along the normal transport pathways of the protein it binds to. A similar assay has been used previously to verify nucleocytoplasmic shuttling of nucleolar proteins (3). A mixture of the affinity-purified anti-Rev MAb 1G10 and Texas red-conjugated dextran (500 kDa) was microinjected into either the nucleus or the cytoplasm of Rev-expressing HeLa/crev cells. The dextran was necessary to control whether the nucleus or the cytoplasm was injected. In Fig. 5A to C the cells were fixed 7 h after

intracellular injection and incubation at 37°C. The dextran was restricted to the nucleoplasm during that period (Fig. 5A). In contrast, the anti-Rev MAb 1G10 was clearly redistributed partly to the nucleolus and partly to the cytoplasm in the course of the incubation (Fig. 5B). The possibility that the redistribution of Rev was an injection or postfixation artifact was excluded by the following experiment. The anti-Rev MAb 1G10 was injected into the HeLa/crev nucleus at 4°C and remained in the nucleus at 4°C when the cells were fixed and examined 7 h later (Fig. 5D to F). In contrast to the cytoplasmic accumulation, the nucleolar accumulation of Rev was often seen in the latter case independently of temperature (data not shown). When the Rev MAb was microinjected into the nuclei of HeLa/crev cells that did not express Rev, the dextran and MAb 1G10 colocalized in the injected compartment for extended periods at 37°C (Fig. 5G to I). Finally, an anti-HIV reverse transcriptase MAb injected into the nuclei of HeLa/crev cells stayed inside the nucleus and was not redistributed like the anti-Rev MAb in parallel experiments (data not shown). When the anti-Rev MAb was injected into the cytoplasm and chased for 7 h at 37°C, nuclear import of Rev took place (Fig. 5G, J, K, and L). Advantage was taken of the fact that multinucleate HeLa/crev cells appeared spontaneously at a low frequency in the culture. In Fig. 5J to L this phenomenon is exemplified. The anti-Rev MAb injected into one of three nuclei (stained with Texas red in Fig. 5J) was exported to the cytoplasm and next imported to the nucleoli of the two other nuclei of the same cell (Fig. 5K).

DISCUSSION

The complex pathway of eukaryotic hnRNA splicing *in vitro* has been examined in detail in nuclear extracts. In a highly ordered succession of events snRNPs and accessory splicing proteins assemble on the intron- and exon-containing hnRNA into a multimolecular structure known as the spliceosome. The process culminates in the release of mRNA, with the exons correctly ligated and the intervening intron sequences removed (reviewed in reference 19). A current hypothesis holds that the mRNA splicing process thus delineated may be compartmentalized in structures known as speckles in the nucleoplasm of living cells (40). The individual components of the *in vitro*-defined spliceosome (snRNPs [5, 25, 27, 48], snRNAs [5, 39], accessory splicing factors [18], poly(A) RNA [7], and specific transcripts [24, 51, 52]) have been visualized as nucleoplasmic speckles by indirect immunofluorescence or *in situ* hybridization. In order to express its condensed genomic information, HIV exploits the host cell RNA splicing machinery and additionally overrides a general paradigm of eukaryotic mRNA expression: that intron-containing RNA is confined to the nucleus. It is conceivable that to interfere with the host cell splicing of HIV RNA, Rev has to be transported from the cytoplasm to the nuclear speckles if this is the site of RNA splicing *in vivo*. In the present study colocalization between Rev and the RNA splicing factor-enriched speckles was found in both a HeLa/crev cell line (Fig. 1) and the lymphoid cell line C8166 infected with the HIV-1 IIIB strain (Fig. 2). The Rev protein shuttled continuously between the nucleoplasmic speckles and the cytoplasm, as demonstrated by two independent assays (Fig. 3 and 4 and Fig. 5). Since Rev can bind to the RRE of the HIV mRNAs it regulates, the results suggest that Rev may be directed to the sites of RNA splicing in the nucleoplasm in order to interfere specifically with splicing of RRE-RNA and mediate transport of such HIV mRNAs to the cytoplasm for translation. In confirmative experiments done in parallel it was shown that Rev performed similarly to the

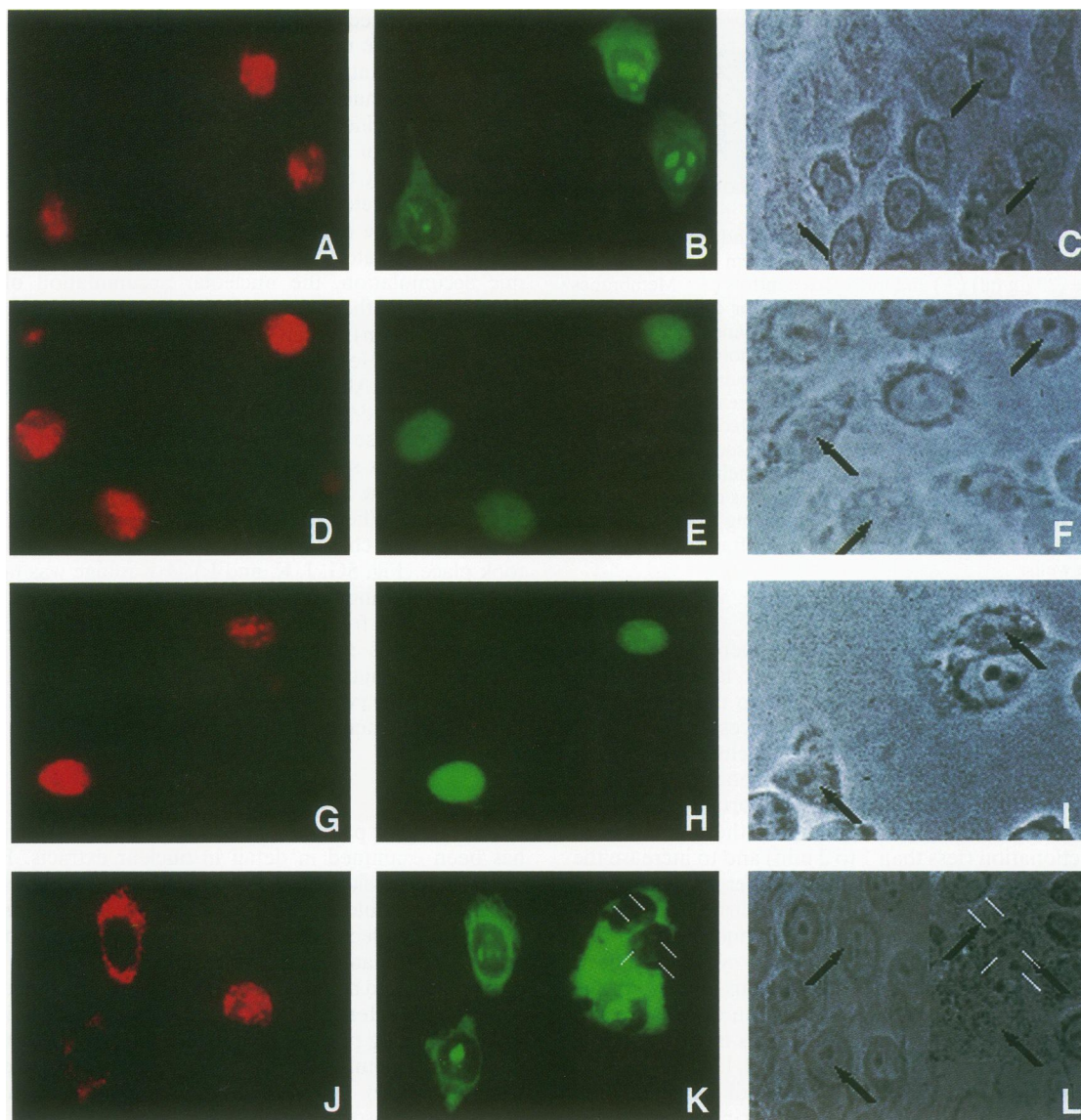


FIG. 5. HeLa/*crev* and HeLa/*cref* cells were grown on coverslips and microinjected with the 1G10 anti-Rev MAb (1 mg/ml) mixed with 1 mg of Texas red-conjugated dextran per ml (500 kDa) (Molecular Probes Inc.) into the nuclei (A to I) or into the cytoplasm (J to L [on the left]) of cells. (A to C and J to L) HeLa/*crev* cells were incubated in complete medium for 7 h at 37°C following microinjection and then fixed in methanol. The anti-Rev MAb was visualized by using FITC anti-mouse immunoglobulin. (D to F) HeLa/*crev* cells were injected and incubated for 7 h at 4°C and then processed together with the other cells (see above). (G to I) HeLa/*cref* cells were microinjected into the nuclei by using the same mixture of the 1G10 anti-Rev MAb and Texas red-dextran. When a HeLa/*crev* cell with three nuclei (upper right in panels J to L) was injected into one nucleus, the Rev MAb was exported to the cytoplasm and imported into the nucleoli (white lines) of the two other nuclei. Arrows in the phase-contrast photographs (C, F, I, and L), microinjected cells.

nucleocytoplasmic shuttle protein hnRNP A1 (Fig. 3D to F and G to I, respectively). Previously, hnRNP A1 has been shown to remain bound to mRNA in the cytoplasm of cells and has been proposed to be part of the host cell mRNA RNP export particle (37). However, Rev displays a nucleolar stage of its subcellular trafficking in contrast to hnRNP A1. It has been suggested that the nucleolar accumulation of Rev might be related to overexpression and nonspecific affinity between Rev and nucleolar components. In this study it was shown for the first time how Rev was distributed in HIV-infected lymphoid cells in situ. Rev exhibited cytoplasmic, perinuclear, nucleolar, and speckled staining in this less artificial system (Fig. 2). Because of an inherent unstable production of Rev in the

methotrexate-selected HeLa/*crev* cells the effect of very different abundance levels of Rev in individual, neighboring cells could be examined. It was apparent that Rev was distributed between the speckles, the nucleolus, and the cytoplasm at all detectable expression levels (Fig. 1G and 3D), arguing against the possibility that the patterns represent overexpression epiphenomena. The Rev patterns did not depend upon the stage of the interphase of the cell cycle and persisted in subclones of the HeLa/*crev* cells (27).

We previously demonstrated that cycloheximide at concentrations sufficient to inhibit new protein synthesis did not affect the subcellular distribution of Rev for periods of 8 to 10 h (27). The pronounced effect of actinomycin D (Fig. 3 and 4) and the

microinjection experiments (Fig. 5) show that the Rev patterns reflect nucleocytoplasmic shuttling of Rev. The transport of Rev in both directions over the nuclear membrane is an active process. The 116-amino-acid Rev protein (apparent molecular size, 21 kDa) is small enough to passively diffuse across the nuclear pore complex. An active transport mechanism is required, however, for the Rev-anti-Rev MAb complex to move across the nuclear pore.

Most studies have found that little unspliced and singly spliced HIV mRNA is exported to the cytoplasm in the absence of Rev (14, 17, 22, 26, 36). In conflicting studies substantial amounts of Rev-dependent RNA appear in the cytoplasm independently of Rev (1, 12). Instead, the predominant effect of Rev was explained by specific loading of such mRNAs on polysomes in the cytoplasm (1, 12). Our experience with cell fractionations (Fig. 4) indicates that possible leakage from the nuclear fraction to the cytoplasmic fraction cannot be ruled out in the latter studies. Aside from that, both Rev-mediated export and cytoplasmic events can be understood as different aspects of the nucleocytoplasmic shuttling of Rev discovered in this study. Different mechanisms have been proposed to account for the Rev-mediated RRE-RNA export. Rev might displace RNA splicing factors from the nascent HIV pre-mRNA (8, 29). Alternatively, Rev might promote specific RNA transport mechanisms or pathways (33). The *in vivo* shuttling of Rev between the speckles enriched in RNA splicing factors and the cytoplasm suggests that the distinction between Rev activity in RNA splicing and RNA transport may be arbitrary.

In the HeLa/*crev* cells (Fig. 1 and 3 to 5) neither the RRE nor HIV proteins other than Rev were made. The Rev protein nevertheless completed its complicated subcellular trafficking and shuttling by direct interaction with host cell components. The RRE was not required to target the Rev protein to the speckles (Fig. 1 and 3) or back into the cytoplasm (Fig. 3 to 5). Very similar distributions of Rev were found in the HeLa/*crev* cells and in the HIV-1-infected C8166 cells (Fig. 2). It is therefore possible that the RRE is not a critical transport determinant of Rev or a putative RRE-Rev complex. The role of Rev may then be to act as a molecular bridge between the RRE-RNA and the host cell transport machinery. The basic domain of Rev is already known to bind to the RRE. Another functional domain of Rev, the leucine-rich activation domain, may bind to a host cell transport factor according to that model. The very recent identification of an RNA sequence from the Mason-Pfizer monkey virus that obviates the need for both Rev and the RRE when it is present *in cis* in otherwise Rev-dependent mRNAs (4) also fits with the notion that Rev is a molecular bridge between the RRE-RNA and a host cell factor. Recently, a host cell factor, eIF5A, that interacts with the Rev activation domain was found and shown to be of functional relevance (43). In a separate publication (48a) we show that, when the activation domain of Rev is mutated in order to generate transdominant negative mutants of Rev, the nuclear export part of the Rev nucleocytoplasmic shuttling is blocked, lending further credence to the hypothesis that Rev binds to a host cell nuclear export factor via its activation domain.

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REFERENCES

1. Arrigo, S. J., and I. S. Y. Chen. 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 *vif*, *vpr* and *env/vpu* 2 RNAs. *Genes Dev.* 5:808-819.
2. Bochnig, P., R. Reuter, P. Bringmann, and R. Lührmann. 1987. A monoclonal antibody against 2,2,7-trimethylguanosine that reacts with intact class U, small nuclear ribonucleoprotein as well as with 7-methylguanosine-capped RNAs. *Eur. J. Biochem.* 168:461-467.
3. Borer, R. A., C. F. Lehner, H. M. Eppenberger, and E. A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* 56:379-390.
4. Bray, M., S. Prasad, J. W. Dubay, E. Hunter, K.-T. Jeang, D. Rekosh, and M.-L. Hammarskjöld. 1994. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proc. Natl. Acad. Sci. USA* 91:1256-1260.
5. Carmo-Fonseca, M., R. Pepperkok, B. S. Sproat, W. Ansorge, M. S. Swanson, and A. I. Lamond. 1991. *In vivo* detection of snRNP-rich organelles in the nuclei of mammalian cells. *EMBO J.* 10:1863-1873.
6. Carmo-Fonseca, M., D. Tollervey, R. Pepperkok, S. M. L. Barabino, A. Merdes, C. Brunner, P. D. Zamore, M. R. Green, E. Hurt, and A. I. Lamond. 1991. Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO J.* 10:195-206.
7. Carter, K. C., K. L. Taneja, and J. B. Lawrence. 1991. Discrete nuclear domains of poly(A)RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.* 115:1191-1202.
8. Chang, D. D., and P. A. Sharp. 1989. Regulation by HIV Rev depends upon recognition of splice sites. *Cell* 59:789-795.
9. Choi, Y. D., and G. Dreyfuss. 1984. Monoclonal antibody characterization of the C proteins of heterogeneous nuclear ribonucleoprotein complexes in vertebrate cells. *J. Cell Biol.* 99:1997-2004.
10. Cochrane, A. W., K. S. Jones, S. Beidas, P. J. Dillon, A. M. Skalka, and C. A. Rosen. 1991. Identification and characterization of intragenic sequences which repress human immunodeficiency virus structural gene expression. *J. Virol.* 62:5305-5313.
11. Cullen, B. R. 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* 56:2498-2501.
12. D'Agostino, D. M., B. K. Felber, J. E. Harrison, and G. N. Pavlakis. 1992. The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of *gag/pol* and *vpu/env* mRNAs. *Mol. Cell. Biol.* 12:1375-1386.
13. Dayton, A. L., E. F. Terwilliger, J. Potz, M. Kowalski, J. G. Sodroski, and W. A. Haseltine. 1988. Cis-acting sequences responsive to the Rev gene product of the human immunodeficiency virus. *J. Acquired Immune Defic. Syndr.* 1:441-452.
14. Emerman, M., R. Vazeux, and K. Peden. 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* 57:1155-1165.
15. Fankhauser, C., E. Izaurralde, Y. Adachi, P. Wingfield, and U. K. Laemmli. 1991. Specific complex of human immunodeficiency virus type 1 Rev and nucleolar B23 proteins: dissociation by the Rev response element. *Mol. Cell. Biol.* 11:2567-2575.
16. Feinberg, M. B., R. F. Jarret, A. Aldovini, R. C. Gallo, and F. Wong-Staal. 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* 46:807-817.
17. Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis. 1989. Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc. Natl. Acad. Sci. USA* 86:1495-1499.
18. Fu, X.-D., and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature (London)* 343:437-441.

19. Green, M. R. 1991. Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annu. Rev. Cell Biol.* **7**:559–599.
20. Green, M. R. 1993. Molecular mechanisms of Tat and Rev. *AIDS Res. Rev.* **3**:41–55.
21. Habets, W. J., M. H. Hoet, B. A. W. De Jong, A. Van der Kemp, and W. J. van Venrooij. 1989. Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally induced mouse monoclonal antibodies. *J. Immunol.* **143**:2560–2566.
22. Hammarskjöld, M.-L., J. Heimer, B. Hammarskjöld, I. Sangwan, L. Albert, and D. Rekosh. 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. *J. Virol.* **63**:1959–1966.
23. Haseltine, W. A. 1991. Regulation of HIV-1 replication, p. 1–42. In W. A. Haseltine and F. Wong-Staal (ed.), *Genetic structure and regulation of HIV-1*. Raven Press, Ltd., New York.
24. Huang, S., and D. L. Spector. 1991. Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes Dev.* **5**:2288–2302.
25. Jiménez-García, L. F., and D. L. Spector. 1993. In vivo evidence that transcription and splicing are coordinated by a recruiting mechanism. *Cell* **73**:47–59.
26. Kalland, K.-H., E. Langhoff, H. J. Bos, H. Göttlinger, and W. A. Haseltine. 1991. Rex-dependent nucleolar accumulation of HTLV-I mRNA. *New Biol.* **3**:389–397.
27. Kalland, K.-H., A. M. Szilvay, E. Langhoff, and G. Haukenes. 1994. Subcellular distribution of human immunodeficiency virus type 1 Rev and colocalization of Rev with RNA splicing factors in a speckled pattern in the nucleoplasm. *J. Virol.* **68**:1475–1485.
28. Kim, S., R. Byrn, J. Groopman, and D. Baltimore. 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential expression. *J. Virol.* **63**:3708–3713.
29. Kjems, J., and P. A. Sharp. 1993. The basic domain of Rev from human immunodeficiency virus type 1 specifically blocks the entry of U4/U6.U5 small nuclear ribonucleoprotein in spliceosome assembly. *J. Virol.* **67**:4769–4776.
30. Lerner, E. A., M. R. Lerner, C. A. Janeway, and J. A. Steitz. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA* **78**:2737–2741.
31. Loewinger, L., and F. McKeon. 1988. Mutations in the nuclear lamin proteins resulting in their aberrant assembly in the cytoplasm. *EMBO J.* **7**:2301–2309.
32. Maldarelli, F., M. A. Martin, and K. Strebel. 1991. Identification of posttranscriptionally active inhibitory sequences in human immunodeficiency virus type 1 RNA: novel level of gene regulation. *J. Virol.* **65**:5732–5743.
33. Malim, M. H., and B. R. Cullen. 1993. Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. *Mol. Cell. Biol.* **13**:6180–6189.
34. Malim, M. H., R. Fenrick, D. W. Ballard, J. Hauber, E. Böhnlein, and B. R. Cullen. 1989. Functional characterization of a complex protein-DNA-binding domain located within the human immunodeficiency virus type 1 long terminal repeat leader region. *J. Virol.* **63**:205–214.
35. Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen. 1988. Immunodeficiency virus rev trans-activator modulates the expression of the viral regulatory genes. *Nature (London)* **335**:181–183.
36. Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen. 1989. The HIV-1 rev transactivator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (London)* **338**:254–257.
37. Piñol-Roma, S., and G. Dreyfuss. 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature (London)* **355**:730–732.
38. Pomerantz, R. J., D. Trono, M. B. Feinberg, and D. Baltimore. 1990. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. *Cell* **61**:1271–1276.
39. Reuter, R., B. Appel, P. Bringmann, J. Rinke, and R. Lührmann. 1984. 5'-terminal caps of snRNAs are reactive with antibodies specific for 2,2,7-trimethylguanosine in whole cells and nuclear matrices. *Exp. Cell Res.* **195**:27–37.
40. Rosbash, M., and R. H. Singer. 1993. RNA travel: tracks from DNA to cytoplasm. *Cell* **75**:399–401.
41. Rosen, C. A., and G. N. Pavlakis. 1990. Tat and rev: positive regulators of HIV gene expression. *AIDS* **4**:499–509.
42. Rosen, C. A., E. Terwilliger, A. Dayton, J. Sodroski, and W. A. Haseltine. 1988. Intragenic cis-acting art-gene-responsive sequences of the human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **85**:2071–2075.
43. Ruhl, M., M. Himmelpach, G. M. Bahr, F. Hammerschmid, H. Jaksche, B. Wolff, H. Aschauer, G. K. Farrington, H. Probst, D. Bevec, and J. Hauber. 1993. Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. *J. Cell Biol.* **123**:1309–1320.
44. Sadaie, M. R., T. Benter, and F. Wong-Staal. 1988. Site-directed mutagenesis of two trans-regulatory genes (tat-III, trs) of HIV-1. *Science* **239**:910–913.
45. Salahuddin, S. Z., P. D. Markham, F. Wong-Staal, G. Franchini, V. S. Kalyanaraman, and R. C. Gallo. 1983. Restricted expression of human T-cell leukemia-lymphoma virus (HTLV) in transformed human umbilical cord blood lymphocytes. *Virology* **129**:51–64.
46. Schwartz, S., B. K. Felber, and G. N. Pavlakis. 1992. Distinct RNA sequences in the *gag* region of human immunodeficiency virus type 1 decrease RNA stability and inhibit expression in the absence of Rev protein. *J. Virol.* **66**:150–159.
47. Sodroski, J., W. C. Goh, C. A. Rosen, A. Dayton, E. Terwilliger, and W. A. Haseltine. 1986. A second post-transcriptional activator gene required for HTLV-III replication. *Nature (London)* **321**:412–417.
48. Spector, D. L. 1990. Higher order nuclear organization: three-dimensional distribution of small nuclear ribonucleoprotein particles. *Proc. Natl. Acad. Sci. USA* **87**:147–151.
- 48a. Szilvay, A. M., K. A. Brokstad, G. Haukenes, and K.-H. Kalland. Nuclear export of the nucleocytoplasmic shuttle protein HIV-Rev is blocked by mutations in its activation domain. Submitted for publication.
49. Szilvay, A. M., S. Nornes, I. R. Haugan, L. Olsen, V. R. Prasad, C. Endresen, S. P. Goff, and D. E. Helland. 1992. Epitope mapping of HIV-1 reverse transcriptase with monoclonal antibodies that inhibit polymerase and RNase H activities. *J. Acquired Immune Defic. Syndr.* **5**:647–657.
50. Terwilliger, E., R. Burghoff, R. Sia, J. Sodroski, and W. Haseltine, and C. Rosen. 1988. The *art* gene product of human immunodeficiency virus is required for replication. *J. Virol.* **62**:655–658.
51. Wang, J., L.-G. Cao, Y.-L. Wang, and T. Pederson. 1991. Localization of pre-mRNA at discrete nuclear sites. *Proc. Natl. Acad. Sci. USA* **88**:7391–7395.
52. Xing, Y., C. V. Johnson, P. R. Dobner, and J. B. Lawrence. 1993. Higher level of organization of individual gene transcription and RNA splicing. *Science* **62**:655–658.