Journal of Cell Science 108, 2811-2823 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The roles of nucleolar structure and function in the subcellular location of the HIV-1 Rev protein

Miroslav Dundr[1], Gregory H. Leno[1], Marie-Louise Hammarskjöld[2], David Rekosh[2], C. Helga-Maria[2] and Mark O. J. Olson[1,*]

[1]Department of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA
[2]Myles H. Thaler Center for AIDS and Human Retrovirus Research, University of Virginia, HSC Box 441, 7-85 Jordan Hall, Charlottesville, VA 22908, USA

*Author for correspondence (e-mail: molson@fiona.umsmed.edu)

SUMMARY

The human immunodeficiency virus 1 (HIV-1) Rev trans-activator protein plays a critical role in the regulation of expression of structural proteins by controlling the pathway of mRNA transport. The Rev protein is located predominantly in the nucleoli of HIV-1 infected or Rev-expressing cells. Previous studies demonstrated that the Rev protein forms a specific complex in vitro with protein B23 which is suggested to be a nucleolar receptor and/or carrier for the Rev protein. To study the role of the nucleolus and nucleolar proteins in Rev function, transfected COS-7 or transformed CMT3 cells expressing the Rev protein were examined for subcellular locations of Rev and other proteins using indirect immunofluorescence and immunoelectron microscopy. One day after transfection the Rev protein was found in most cells only in the nucleolar dense fibrillar and granular components where it colocalized with protein B23. These were designated class 1 cells. In a second class of cells Rev and B23 accumulated in the nucleoplasm as well as in nucleoli. Treatment of class 1 cells with actinomycin D (AMD) under conditions that blocked only RNA polymerase I transcription caused Rev to completely redistribute from nucleoli to the cytoplasm. Simultaneously, protein B23 was partially released from nucleoli, mostly into the nucleoplasm, with detectable amounts in the cytoplasm. In cells recovering from AMD treatment in the presence of cycloheximide Rev and B23 showed coincident relocation to nucleoli. Class 2 cells were resistant to AMD-induced Rev redistribution. Selective inhibition of RNA polymerase II transcription by α-amanitin or by DRB did not cause Rev to be released into the cytoplasm suggesting that active preribosomal RNA transcription is required for the nucleolar location of Rev. However, treatment with either of the latter two drugs at higher doses and for longer times caused partial disruption of nucleoli accompanied by translocation of the Rev protein to the cytoplasm. These results suggest that the nucleolar location of Rev depends on continuous preribosomal RNA transcription and a substantially intact nucleolar structure.

Key words: HIV-1 Rev protein, nucleolus, B23/nucleophosmin

INTRODUCTION

The replication cycle of human immunodeficiency virus type 1 (HIV-1) takes place at several levels in the nucleus and cytoplasm. At the initial stages of infection the primary viral transcript is multiply spliced into 2-kb transcripts encoding the regulatory proteins Tat, Rev and Nef. After the synthesis of sufficient levels of these proteins the host cell generates singly spliced 4-kb mRNAs encoding Vif, Vpr, Vpu, Env and unspliced 9-kb mRNAs encoding Gag and Pol. The HIV-1 *trans*-activator Rev is essential for the cytoplasmic accumulation and expression of singly spliced or unspliced viral mRNAs (Emerman et al., 1989; Felber et al., 1989; Hammarskjöld et al., 1989; Malim et al., 1989). The regulation of splicing takes place through the direct interaction between Rev and a *cis*-acting stem-loop target sequence called the Rev response element (RRE) present in these mRNAs (Rosen et al., 1988; Daly et al., 1989; Hadzopoulou-Cladaras et al., 1989; Tiley et al., 1992).

The Rev protein is imported into the nucleus and accumulates predominantly in the nucleoli of expressing cells. A highly basic stretch in the Rev sequence located between positions 35 and 50, has been identified as a nucleolar targeting signal (NOS) (Kubota et al., 1989; Malim et al., 1989; Cochrane et al., 1990). This domain is also required for the highly specific binding of the Rev protein to the viral RRE (Kjems et al., 1991; Malim and Cullen, 1991). The two regions flanking this basic sequence are essential for the multimerization of Rev monomers on the RRE (Olsen et al., 1990; Malim and Cullen, 1991; Zapp et al., 1991). A short domain close to the C-terminus of Rev which is rich in characteristically spaced leucine residues has been hypothesized to mediate *trans* activation by protein-protein interactions with host cell factor(s) critical for the export of viral pre-mRNAs (Malim et al., 1989; Malim and Cullen, 1991).

Several hypotheses have been offered regarding the mechanism by which the Rev-RRE interaction and *trans* acti-

vation by the Rev protein culminate in the expression of specific viral proteins. It has been proposed that the Rev protein induces the translocation of intron-containing mRNAs from the nucleus to the cytoplasm by inhibition of splicing or spliceosome assembly (Chang and Sharp, 1989; Kjems et al., 1991; Kjems and Sharp, 1993). Alternatively, it has been suggested that Rev directly promotes the export of RRE-containing pre-mRNAs to the cytoplasm independently of any inhibitory effect on the splicing of pre-mRNA (Felber et al., 1989; Malim and Cullen, 1993; Fischer et al., 1994). There is evidence that Rev interacts with components of the splicing machinery to allow export of the mRNA into the cytoplasm before splicing occurs (Lu et al., 1990; Hammarskjöld et al., 1994). Rev may do this by counteracting the elements in the HIV-1 mRNA that promote retention of the unspliced RNA in the nucleus. In fact, this can be accomplished by factors other than the Rev protein. For example, a 219-nucleotide sequence from the Mason-Pfizer monkey virus can substitute for Rev and the RRE to promote mRNA export and expression of HIV-1 structural proteins (Bray et al., 1994). Finally, it has been suggested that there is a critical cytoplasmic role for Rev in the activation of viral mRNA translation (Arrigo and Chen, 1991; DíAgostino et al., 1992; Campbell et al., 1994).

In an effort to determine which nuclear proteins interact with the Rev protein it was discovered by Fankhauser et al. (1991) that nucleolar protein B23 forms a stable complex with the Rev protein in vitro, which may be dissociated by the RRE-containing RNA. Similarly, protein B23 was identified as the host protein for binding the functional analog of the Rev protein, the Rex protein of human T-cell leukemia virus type I (HTLV-I) (Adachi et al., 1993). The Rex protein and probably the Rev protein interact through their basic NOS sequences with two highly acidic regions of protein B23. Because of these findings it has been suggested that protein B23 is the possible nucleolar acceptor and/or transporter for Rev.

The accumulation of the Rev protein in nucleoli is of potential importance either as part of a transport mechanism or as a temporary site of storage. Protein B23 appears to be at least partially responsible for the nucleolar location of Rev. Therefore, these studies were initiated to determine the extent of colocalization of the two proteins in Rev expressing cells under various conditions and whether nucleolar localization was dependent on nucleolar function. It was found that Rev and B23 are localized together in the dense fibrillar and granular components of the nucleolus. In addition, continued synthesis of preribosomal RNA appears to be essential for nucleolar localization of both proteins.

MATERIALS AND METHODS

Rev-expressing plasmid

Plasmid pCMVrev contains HIV-1 *rev* cDNA positioned between a CMV promoter and β-globin splice and poly(A) signals as previously described (Lewis et al., 1990).

Cell cultures

The COS-7 and CMT3 monkey cell lines (Gerard and Gluzman, 1985) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco). The COS-7 cells grown on coverslips were transfected using the DEAE-dextran/chloroquine method (Cullen, 1987). Cell lines constitutively expressing the HIV-1 Rev protein were obtained after transfection of monkey CMT3 cells (Gerard and Gluzman, 1985) with the plasmid pCMVrev together with a plasmid pHyg that confers hygromycin resistance. Plasmid pCMVrev (previously known as pRev1) contains the HIV-1 *rev* gene under control of the simian CMV immediate early promoter/enhancer (Smith et al., 1990). Plasmid pHyg was a kind gift from Dr J. Yates (Sugden et al., 1985). CMT3 cells were transfected using the calcium phosphate method and hygromycin resistant cells were selected in hygromycin B containing medium (200 μg/ml). Resistant colonies were screened for the Rev expression by immunofluorescence and immunoblotting using a Rev specific serum.

Antibodies

The Rev protein was detected by a mouse monoclonal antibody (mAb, IgG1) or a rabbit polyclonal antibody (American Biotechnologies) at a dilution of 1:500. In immunoelectron microscopy experiments the Rev protein was detected on sections using a rabbit polyclonal antibody against the C-terminal end of the protein. Human monospecific autoimmune sera, the V11 serum specific for a 70 kDa antigen of the fibrillar centers, and the serum G04 specific for a 52 kDa antigen of the granular components were used at a dilution of 1:150 (kindly provided by Dr D. Hernandez-Verdun), and the serum specific for the fibrillarin (kindly provided Dr R. L. Ochs) was used at a dilution of 1:600. Protein B23 was detected either by polyclonal or monoclonal antibodies. The polyclonal antiserum (prepared by American Qualex, LaMirada, CA) was raised against rat recombinant protein B23.1 purified by the procedure described by Umekawa et al. (1993). The monoclonal antibody was a generous gift from Dr P. K. Chan. The polyclonal and monoclonal antibodies were used at dilutions of 1:400 and 1:500, respectively.

Except for time course experiments, the transiently transfected COS-7 cells were fixed seventeen to fifty hours after transfection with 3% paraformaldehyde in PBS (136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes on ice. The cells were washed extensively with PBS supplemented with 1% BSA. The cells were incubated with the primary antibody for 35 minutes at room temperature, washed four times in PBS for 4 minutes each time, and incubated with a fluorescein- or Texas Red-labeled secondary antibody (Amersham) for 30 minutes at room temperature. The cells were washed with PBS extensively, six times for 4 minutes, briefly in H_2O and ethanol, air dried and mounted on the slides with Mowiol (Calbiochem) containing 1 mg/ml *p*-phenylenediamine.

In some experiments cells were treated with either 0.04 μg or 0.5 μg/ml of actinomycin D (AMD) (Sigma), 1-40 μg/ml of α-amanitin (Sigma), 30 μg or 50 μg/ml of 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) (Sigma) to selectively inhibit RNA transcription. In addition, 20 μg/ml of cycloheximide (Sigma) was included to inhibit protein synthesis.

Fluorescence microscopy

The samples were examined using a laser scanning confocal microscope (Noran) with a Nikon 100×/1.3 N.A. objective. For double labeling, samples were subjected to excitation wavelengths of 488 nm (fluorescein) or 529 nm (Texas Red) from an argon-ion laser. The confocal images for each fluorochrome were recorded independently and photographed using Kodak T-Max 400 film with a digital palette (Polaroid).

Ultrastructural immunocytochemistry

Rev-expressing CMT3 cells were fixed with 8% paraformaldehyde in 200 mM Pipes (pH 7.0) containing 5 mM $MgCl_2$ for 2 hours and then in 0.01% glutaraldehyde in the same buffer for 2 minutes. The cells were washed in PBS, collected by scraping and centrifuged. The cell pellet was initially embedded in 5% gelatin in PBS, dehydrated in

increasing concentrations of ethanol and finally embedded in Lowicryl K4M. Polymerization was performed under long-wavelength UV light (2× 15 W, Ted Pella) for 4 days at −20°C and for 2 days at room temperature. Ultrathin sections were placed on carbon-Parlodoin-coated copper grids. The sections were incubated with primary antibodies for 2-4 hours. After washing with PBS, the grids were incubated with goat anti-rabbit IgG conjugated to 10 nm gold particles. After washing with PBS and water, the grids were stained with 5% aqueous uranyl acetate.

Consecutive double labeling for Rev protein and protein B23 was performed according to the method of Slot and Geuze (1984). Briefly, the sections were incubated with rabbit anti-Rev antibody, and 10 nm Protein A-gold (BioCell Res. Lab.) After washing in PBS the sections were incubated with free Protein A (0.1 mg/ml) in PBS for 10 minutes to ensure that all Protein A binding sites were saturated. The grids were then incubated with rabbit anti-B23 antibody, and 5 nm Protein A-gold. Alternatively, the sections were immunolabeled with anti-B23 antibody and 10 nm Protein A-gold first, and anti-Rev second. As a control, free sections were labeled with anti-Rev protein antibody on one side of the sections and then labeled with anti-B23 antibody on the other side of the sections.

RESULTS

Subcellular location of the Rev protein

Previous morphological studies have shown that the Rev protein is imported to the nucleus and accumulates in the nucleolus (Cullen et al., 1988; Cochrane et al., 1990). To examine the role of the nucleolus in Rev function we used double immunofluorescence microscopy employing anti-Rev antibodies and a series of antibodies against nucleolar proteins to determine the specific location of the Rev protein relative to the locations of those proteins in subcellular structures. In addition, immunoelectron microscopy employing anti-Rev and anti-B23 antibodies was used to obtain a higher level of resolution in the localization studies.

The Rev protein may utilize the system for import of proteins into the nucleolus (Cullen et al., 1988; Rosen et al., 1988), which should make the nucleolus the initial target for Rev imported into the nucleus. This was suggested by experiments with COS-7 cells transiently transfected with a wild-type Rev expression plasmid. In time course experiments the first detectable amount of Rev in these cells was found in nucleoli less than 3 hours after transfection (time zero for the expression of Rev protein was defined as the moment when the DNA/DEAE-dextran solution was removed). However, substantial amounts of Rev were detectable in the nucleoplasm of these cells with longer times in culture. Although Rev was predominantly localized in nucleoli in most cells at early times after transfection (designated class 1 cells; Fig. 1A,B,C), the pattern of Rev location changed with the gradual accumulation of the Rev protein. Cells accumulating the Rev protein at similar levels in the nucleoplasm and in nucleoli were desig-

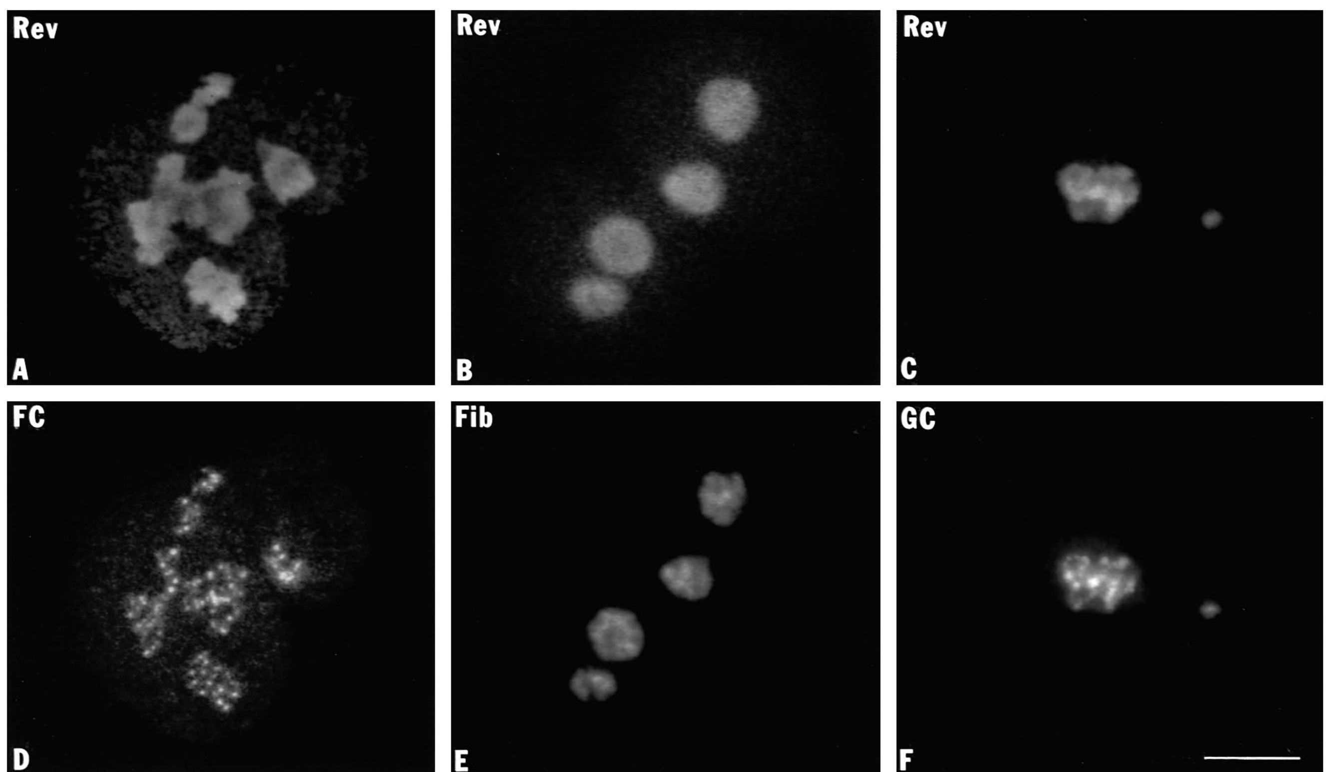

Fig. 1. Localization of the Rev protein and markers of nucleolar subcomponents in class 1 Rev-expressing cells. COS-7 cells transfected (A,D) or line A5.9 CMT3 cells transformed (B,C,E,F) with the pCMVrev plasmid were subjected to double immunofluorescence microscopy. In this and subsequent figures the upper panel of each pair of light micrographs shows localization of the Rev protein and the lower panel shows localization of another antigen in the same optical section. The Rev protein was localized using a monoclonal anti-Rev antibody. In cells having the first class of accumulation the Rev protein is localized to intact nucleoli (A-C) and not coincident with the distinct punctate distribution of the FCs (D) labeled with V11 autoantibody. The nucleolar distribution of the Rev protein (B) colocalized with the S4 serum against the fibrillarin, predominantly localized in the DFC (E) and with the G04 autoantibody specifically recognized the GC (F). Bar, 10 μm.

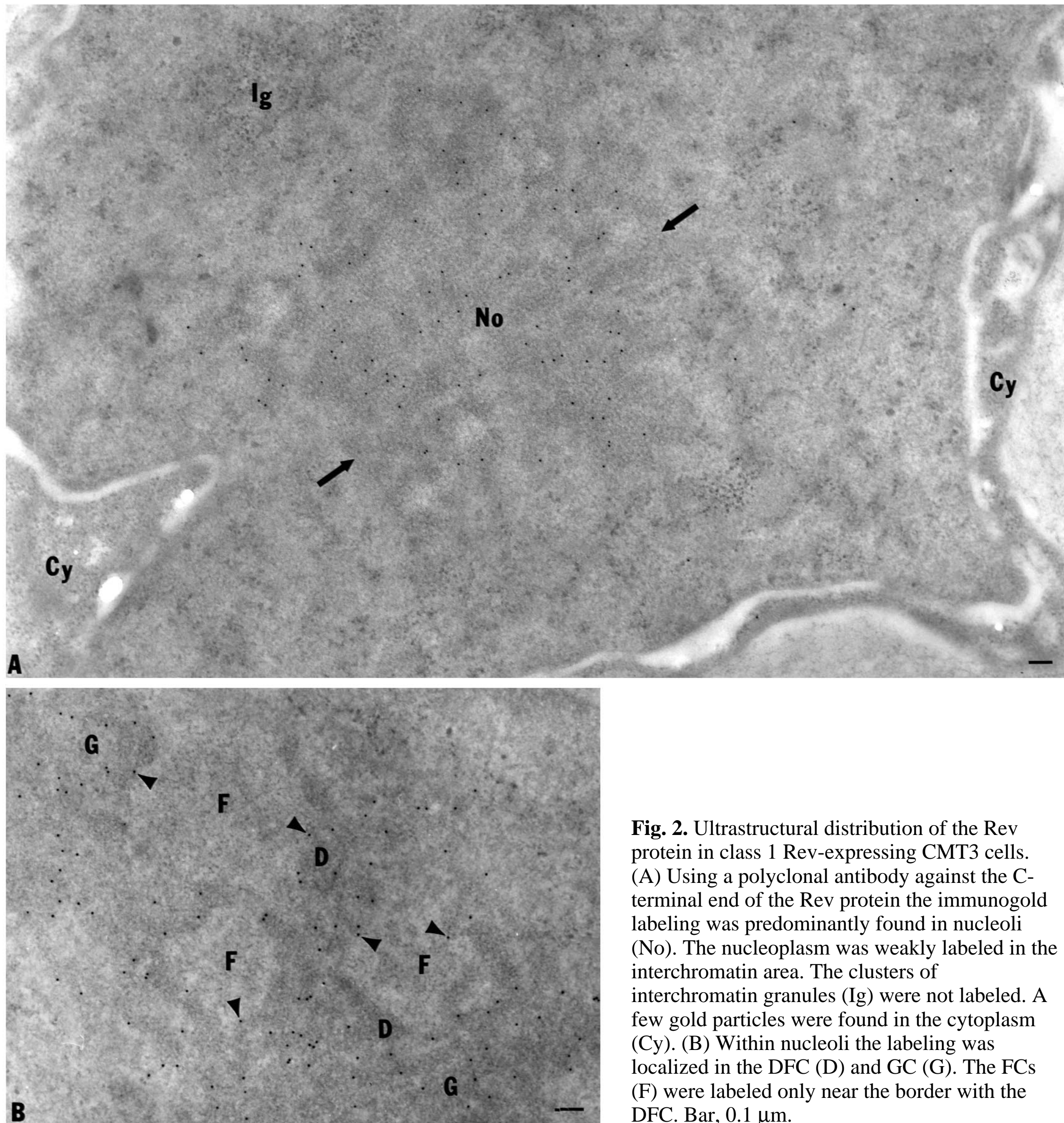

Fig. 2. Ultrastructural distribution of the Rev protein in class 1 Rev-expressing CMT3 cells. (A) Using a polyclonal antibody against the C-terminal end of the Rev protein the immunogold labeling was predominantly found in nucleoli (No). The nucleoplasm was weakly labeled in the interchromatin area. The clusters of interchromatin granules (Ig) were not labeled. A few gold particles were found in the cytoplasm (Cy). (B) Within nucleoli the labeling was localized in the DFC (D) and GC (G). The FCs (F) were labeled only near the border with the DFC. Bar, 0.1 μm.

nated class 2 cells. The transformed CMT3 A5.9 cell line contained mostly class 1 cells.

To more precisely determine the location of the Rev protein in the Rev-expressing CMT3 cells, immunoelectron microscopy was performed using a polyclonal antibody recognizing the C-terminal end of the Rev protein. The ultrastructural studies confirmed the predominant nucleolar location of the Rev protein in class 1 CMT3 cells (Fig. 2A). The nucleoplasm was only weakly labeled, with the signal for Rev randomly dispersed over the interchromatin space. Some weak labeling was also seen at the peripheral regions of the clusters of interchromatin granules (Igs), but not in their interiors. Coiled bodies and perichromatin granules were not labeled.

At longer times after transfection there were increased numbers of class 2 cells which showed the presence of the Rev protein in the nucleoplasm, mainly in the perinuclear area (Fig. 3A,C). The intensities of labeling of the Rev protein in the nucleoli and in the nucleoplasm were approximately the same. In these cells, nucleolar localization of the Rev protein prevailed in the peripheral parts of the nucleoli (Fig. 3A,B,C). In a majority of the cells the nucleoli exhibited normal sizes and compact shapes, but in a smaller number of the cells the nucleoli were enlarged, possibly by nucleolar fusion (data not shown). The nucleolar fibrillar centers (FC) exhibited more distinct punctate patterns with smaller volumes and their numbers were increased in these cells (Fig. 3D, small arrows). In a small number of cells the nucleoli were highly disrupted or could not be seen at all. Since these cells were very rare they were not given a separate classification.

At the ultrastructural level in class 2 Rev-expressing cells the Rev protein was localized in the nucleoplasm and the cytoplasm as well as in the nucleoli (Fig. 4). The nucleoli

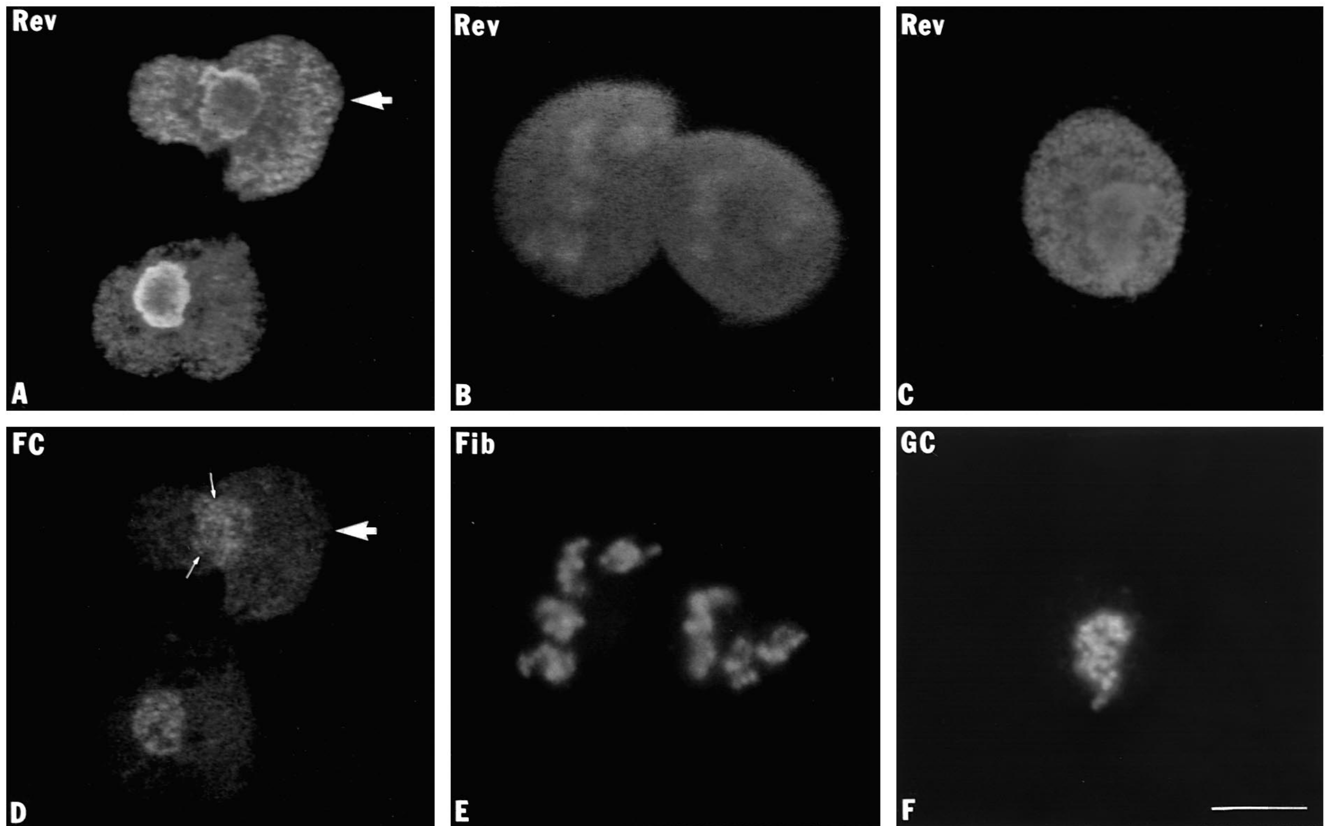

Fig. 3. The subcellular localization of Rev protein in class 2 Rev-expressing cells. Transfected COS-7 cells expressing Rev in the second class of Rev accumulation were selected (A, large arrow; B,C). In class 2 cells the Rev distribution in nucleoli is the same as in class 1 except there is an increasing level of Rev protein in the nucleoplasm, mainly in the perinuclear area (A,B,C) as seen using the anti-Rev monoclonal antibody. The FCs show more distinct punctate patterns with smaller volumes and their numbers are increased (D, small arrows). Immunostaining with anti-fibrillarin serum (D) and anti-GC marker (E) present more distinct patterns of labeling. Bar, 10 μm.

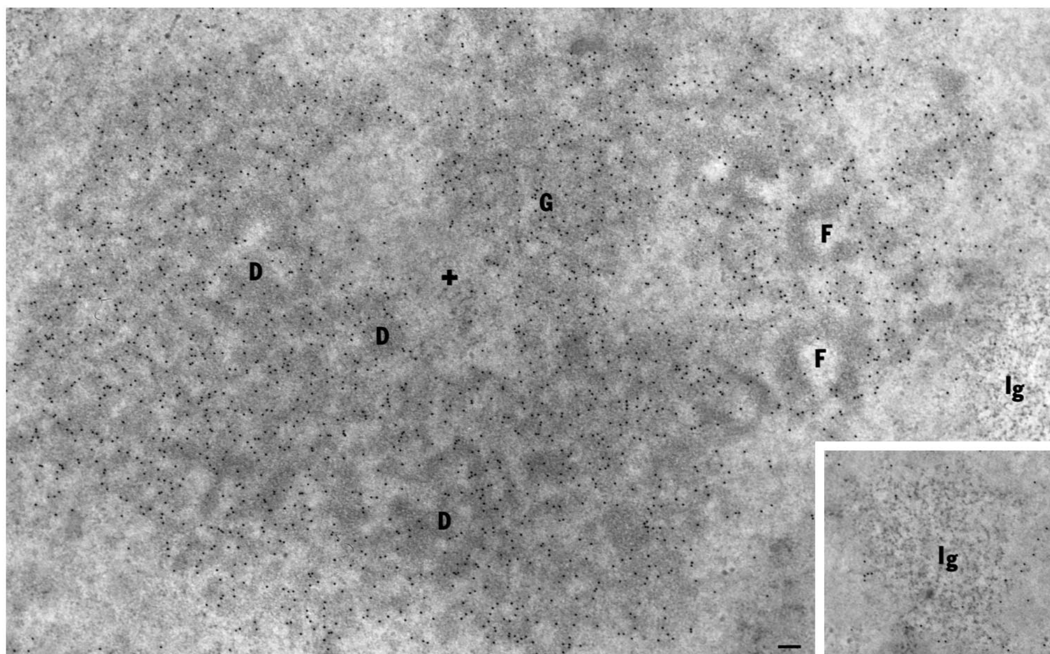

Fig. 4. Ultrastructural distribution of the Rev protein in class 2 Rev-expressing CMT3 cells. Immunogold labeling was performed as for Fig. 2. In class 2 cells the Rev protein was localized in the nucleoplasm and the cytoplasm as well as in the nucleoli. The nucleoli exhibited a greatly increased amount of labeling in the DFC (D) and the GC (G) compared to class 1 cells (see Fig. 2). The nucleoli showed reticular patterns with increased numbers of small FCs (F) and a much higher abundance of DFCs. Parts of the DFCs and GCs were devoid of labeling (cross). The clusters of interchromatin granules (Ig) were labeled only peripherally (inset). Bar, 0.1 μm.

showed reticular patterns with increasing numbers of small FCs and a much higher abundance of dense fibrillar components (DFCs).

The nucleoplasmic labeling of the Rev protein was frequently present in clusters located in the interchromatin space. Some gold particles were present over the periphery of

condensed chromatin. The clusters of Igs were labeled only peripherally (Fig. 4, inset), with the signal for Rev inside the Ig present only rarely. This distribution of Rev at the periphery of the Igs is similar to that of nascent messenger RNAs (Huang et al., 1994; Hendzel and Bazett-Jones, 1995). Perichromatin granules were not labeled. The cytoplasmic signal for the Rev protein was dispersed essentially uniformly over the cytoplasm.

Fig. 5 shows the percentage of cells in two classes of Rev accumulation 22 hours and 44 hours after transfection with the Rev-expressing plasmid. The relative number of class 1 cells was greater than 81% after 22 hours, suggesting an initial preferred location of the Rev protein in the nucleolus. However, after 44 hours the proportion of class 1 cells decreased to 54% and the number of class 2 cells increased to 46%, compared to 19% after 22 hours. This suggests a continuing process of Rev protein accumulation in the nucleus with time.

Subnucleolar location of the Rev protein

To determine the subnucleolar location of the Rev protein we employed indirect immunofluorescence confocal laser microscopy using specific antibodies for three distinct nucleolar structures, the FCs, the DFCs and the granular components (GC). The Rev protein was not entirely coincident with autoimmune serum V11 specific for a 70 kDa antigen of the FC (Fig. 1A,D). However, the Rev protein colocalized with the antibody against fibrillarin, a 34 kDa protein specifically localized in the DFC (Fig. 1B,E) and with autoimmune serum G04 specific for a 52 kDa antigen of the GC (Fig. 1C,F). Both of these Rev-positive nucleolar components are involved in the metabolism of ribosomal RNA (rRNA): the DFC is associated with early events in rRNA transcription and processing and the GC with the maturation of preribosomal subunits. Essentially

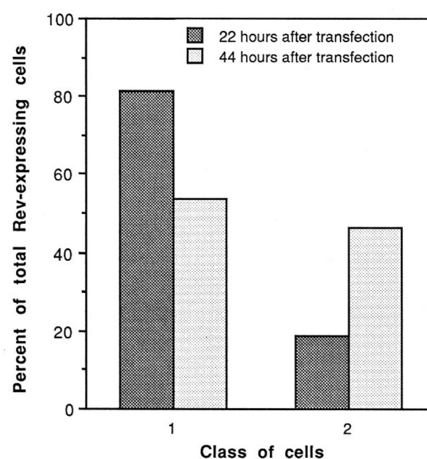

Fig. 5. Distribution of COS-7 cells in class 1 and 2 at 22 hours and 44 hours after transfection with the Rev-expressing plasmid. The numbers of cells in each of the two classes as described in the text and for Figs 1-4 were determined out of a total of 1,200 cells counted for each time point. The data are represented as the percentage of cells of a given class out of the total Rev-expressing cells for each time point.

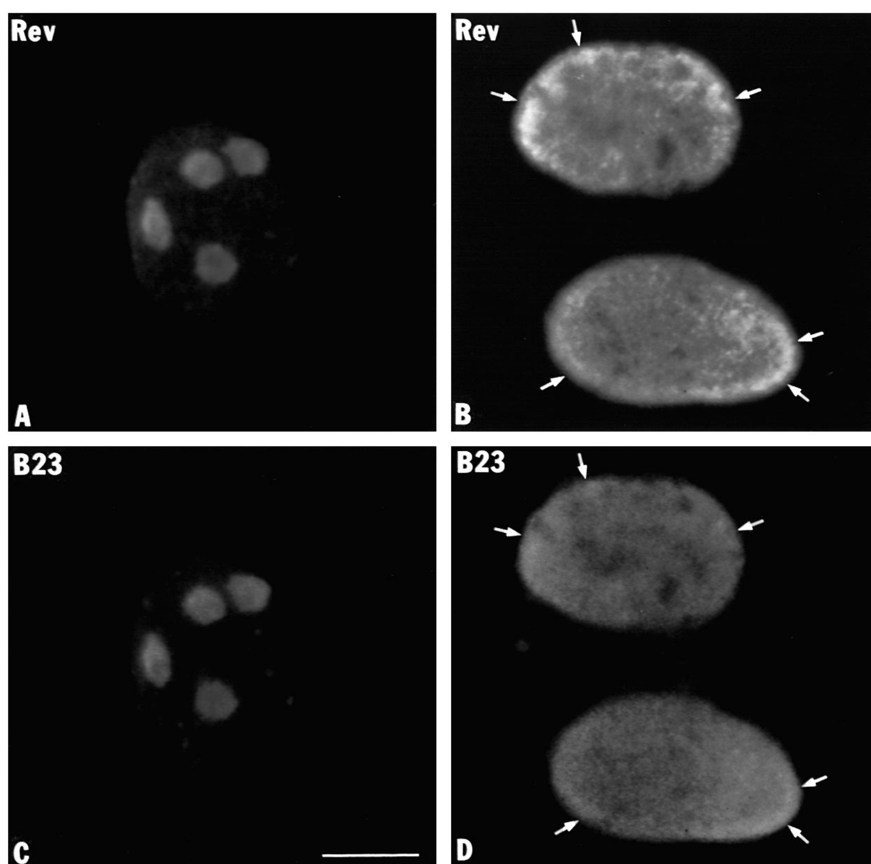

Fig. 6. Protein B23 generally colocalizes with the Rev protein in nucleoli of Rev-expressing cells. The immunofluorescent staining of the Rev protein in nucleoli (A) using a polyclonal anti-Rev antibody colocalizes with protein B23 visualized by a monoclonal anti-B23 antibody in class 1 Rev-expressing CMT3 cells (C). In class 2 COS-7 cells the Rev protein is mainly localized in the perinuclear area (B, arrows) where it colocalizes with protein B23 (D, arrows). Bar, 10 μm.

the same pattern of Rev staining of the DFC and GC was observed in CMT3, a stable Rev-expressing cell line, although the transiently transfected COS-7 cells exhibited a higher level of intensity of staining for Rev than the stable Rev-expressing line. Thus, the Rev protein was shown to be associated with the nucleolar elements containing preribosomal particles in the process of assembly.

At the ultrastructural level of class 1 cells, the Rev protein was concentrated in the DFC and the GC (Fig. 2B). A few gold particles were seen in the FC, but these were only at the border with the DFC (Fig. 2B, arrowheads), where rRNA transcription is believed to take place (Dundr and Raška, 1993). In class 2 cells the nucleoli exhibited progressively higher amounts of the Rev protein in the DFC and the GC (Fig. 4). Rev protein labeling was occasionally absent in parts of the DFCs and GCs (Fig. 4).

Protein B23 generally colocalizes with the Rev protein in nucleoli

To better understand the possible interaction between Rev and B23 we used double labeled indirect immunofluorescence staining to localize both proteins in transiently transfected COS-7 cells and stably transformed Rev-expressing CMT3 cells. In class 1 cells the nucleolar distribution of protein B23 was generally the same as that of the Rev protein (Fig. 6A,C) and the B23 distribution was essentially the same as that of control nontransfected COS-7 cells. In other words, the presence of the Rev protein did not change the nucleolar pattern of B23 staining. However, with increasing localization of Rev in the nucleoplasm we also observed increasing B23 staining in the nucleoplasm (data not shown). In the cells with a high level of Rev protein accumulation, protein B23 was predominantly redistributed to the area near the nuclear envelope where it colocalized with the Rev protein (Fig. 6B, D, arrows). A lower amount of B23 was visible in the more uniformly stained nucleoplasm, but nucleoli were either unlabeled or very weakly labeled (Fig. 6D).

At the ultrastructural level, using consecutive double immunolabeling with different sizes of gold particles, the Rev protein and protein B23 were largely coincidental in location in nucleoli (Fig. 7). Both proteins colocalized in the DFC and the GC; however, in the GC there was a much higher ratio of protein B23 to Rev. This is probably caused by a higher abundance of protein B23 in the GC than in the DFC and vice versa for the Rev protein. This was also observed using smaller gold particles for Rev detection which gave a higher intensity of labeling than the larger gold particles (Slot and Geuze, 1984). In class 1 Rev-expressing cells the nucleoli exhibited some relatively large areas of GCs which were not labeled for either protein (Fig. 7, cross). In non-Rev-expressing cells protein B23 was almost uniformly distributed over the GC (not shown).

Effects of actinomycin D on the subcellular location of the Rev protein

Because the Rev protein is initially targeted to the nucleoli of transfected cells, we attempted to determine if rRNA transcription was necessary for its nucleolar localization. Approximately 17-24 hours after transfection with the Rev-expressing plasmid the COS-7 cells were incubated in medium supplemented with either 0.5 μg/ml or 0.04 μg/ml of the transcription inhibitor actinomycin D (AMD). The higher dose of AMD results in a rapid block of rRNA transcription and possibly partially inhibited activity of RNA polymerases (pols) II and III (Perry and Kelley, 1968, 1970). However, low doses of AMD selectively inhibit rRNA synthesis and do not detectably affect RNA pols II and III (Perry and Kelley, 1968, 1970). In time course experiments with the higher dose there was significant redistribution of Rev from the nucleoli and the nucleoplasm to the cytoplasm within 45 minutes in class 1 cells and complete movement of Rev to the cytoplasm after 50 minutes of treatment. In a very small number of cells there was a very weak signal for Rev in the nucleolar body (data not shown). The nucleoli exhibited a partially segregated pattern; i.e. the FCs were not completely fused into a single region.

In time course experiments with the low dose of AMD (0.04 μg/ml) we observed significant relocation of the Rev protein from nucleoli to cytoplasm after 135 minutes (Fig. 8A). In cells so treated, nucleolar structure exhibited a segregated pattern as detected by localization of fibrillarin (Fig. 8D). Experiments with AMD performed in the presence of cycloheximide (20 μg/ml), which blocked protein synthesis de novo, showed essentially the same effect (data not shown) indicating the shift in location was not due to accumulation of newly synthesized protein. The Rev protein in class 2 cells was insensitive to the effect of AMD seen with class 1 cells with either the high or low dose; in these cells the Rev protein remained in the peripheral parts of the nuclei and did not move to the cytoplasm (Fig. 8C).

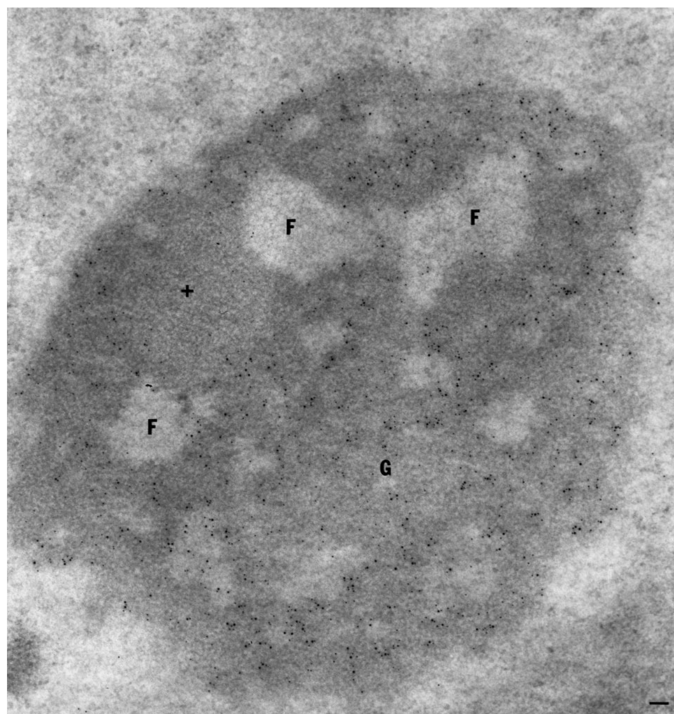

Fig. 7. Colocalization of the Rev protein and protein B23 in nucleoli of class 1 Rev-expressing CMT3 cells. The Rev protein (10 nm gold particles) predominantly colocalized with the protein B23 (5 nm gold particles) in the DFC and the GC. The intensity of labeling for protein B23 was greater than the labeling of the Rev protein. Notice that parts of the GCs (cross) are not labeled for either protein. Bar, 0.1 μm.

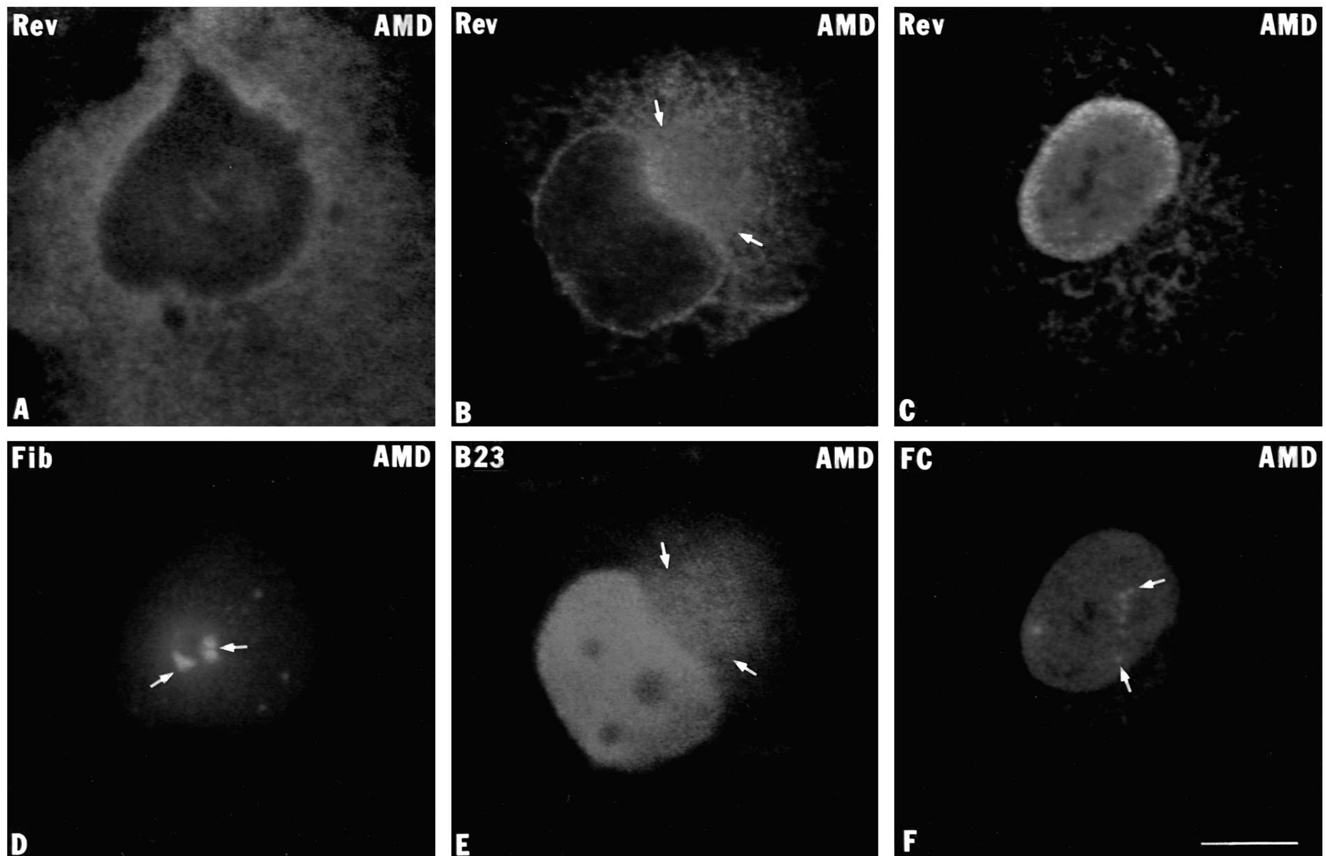

Fig. 8. Effects of AMD treatment on the subcellular location of the Rev protein in Rev-expressing cells. Transformed CMT3 cells (A,B,D,E) or transfected COS-7 cells (C,F) were treated with actinomycin D at two different doses and observed by fluorescence microscopy at various times. The Rev protein is translocated from the nucleoli and the nucleoplasm to the cytoplasm after AMD treatment (0.04 μg/ml) for 135 minutes (A). The nucleoli exhibit segregated patterns for fibrillarin staining (D, arrows). After AMD treatment (0.5 μg/ml) for 50 minutes protein B23 is redistributed to the peripheral region of segregated nucleoli and the nucleoplasm with substantial amounts detectable in the cytoplasm (E, arrows) where it colocalizes with translocated Rev protein (B, arrows). Class 2 cells (C) do not exhibit translocation of the Rev protein from the nuclei to the cytoplasm after AMD treatment treatment (0.5 μg/ml) for 50 minutes. The segregated nucleoli after AMD treatment are visible using the marker for FC (F, arrows). Bar, 10 μm.

After AMD treatment (0.5 μg/ml) of Rev-expressing cells for 50 minutes nucleoli were nearly devoid of protein B23 except for small amounts in peripheral parts corresponding to the segregated region of the GCs (Fig. 8E). The other parts of segregated nucleoli were largely negative for protein B23. Most of protein B23 was redistributed to the nucleoplasm but there was also a significant level of B23 in the cytoplasm, where it colocalized to a large extent with the redistributed Rev protein (Fig. 8B,E, arrows). The cytoplasmic level of B23 was detectable only by using polyclonal anti-B23 antibody: the monoclonal anti-B23 antibody did not show cytoplasmic labeling except in mitotic cells. With longer times of AMD treatment the concentration of protein B23 in the peripheral parts of nucleoli decreased: after 2.5 hours almost none was detected (data not shown).

Treatment of Rev-expressing cells with a low dose of AMD, which selectively blocks RNA pol I transcription, suggested that Rev protein accumulation in nucleoli depends on active rRNA transcription level. In contrast, the findings of Meyer and Malim (1994) suggested that the accumulation of Rev in the nucleoli during interphase was dependent on the activity of RNA pol II because treatment with DRB at 100 μM (31.9 μg/ml) for 3 hours led to the relocalization of wild-type Rev protein to the cytoplasm. It has been shown previously that treatment of cells with DRB causes the nucleoli to reversibly disperse into extended beaded strands (‘necklaces’) throughout the nucleoplasm without inhibition of RNA pol I transcription (Scheer et al., 1984; Scheer and Benavente, 1990). After DRB treatment (50 μg/ml) of our Rev-expressing cells for 90 minutes the Rev protein was present in nucleoli which had dispersed distributions of FC markers (data not shown). After 3 hours incubation with DRB the cells showed further dispersion of nucleolar structure as indicated by distinct extended strands of FC markers: in these nucleoli the Rev protein was somewhat more dispersed than the FC markers (Fig. 9A,D). Continuing DRB treatment for 4 hours caused a significant redistribution of the Rev protein from nuclei to cytoplasm (Fig. 9B,C) with highly dispersed nucleolar necklaces in the nucleoplasm visualized by the anti-FC antibody (Fig. 9E). Similarly, a marker for the GC showed a very high level of dispersion through the nucleoplasm, but it was still partly concentrated around the dispersed nucleolar necklaces (Fig. 9F). Similarly, protein B23 was distributed along extended nucleolar beaded strands and in the nucleoplasm (data not shown). In a small percentage of cells

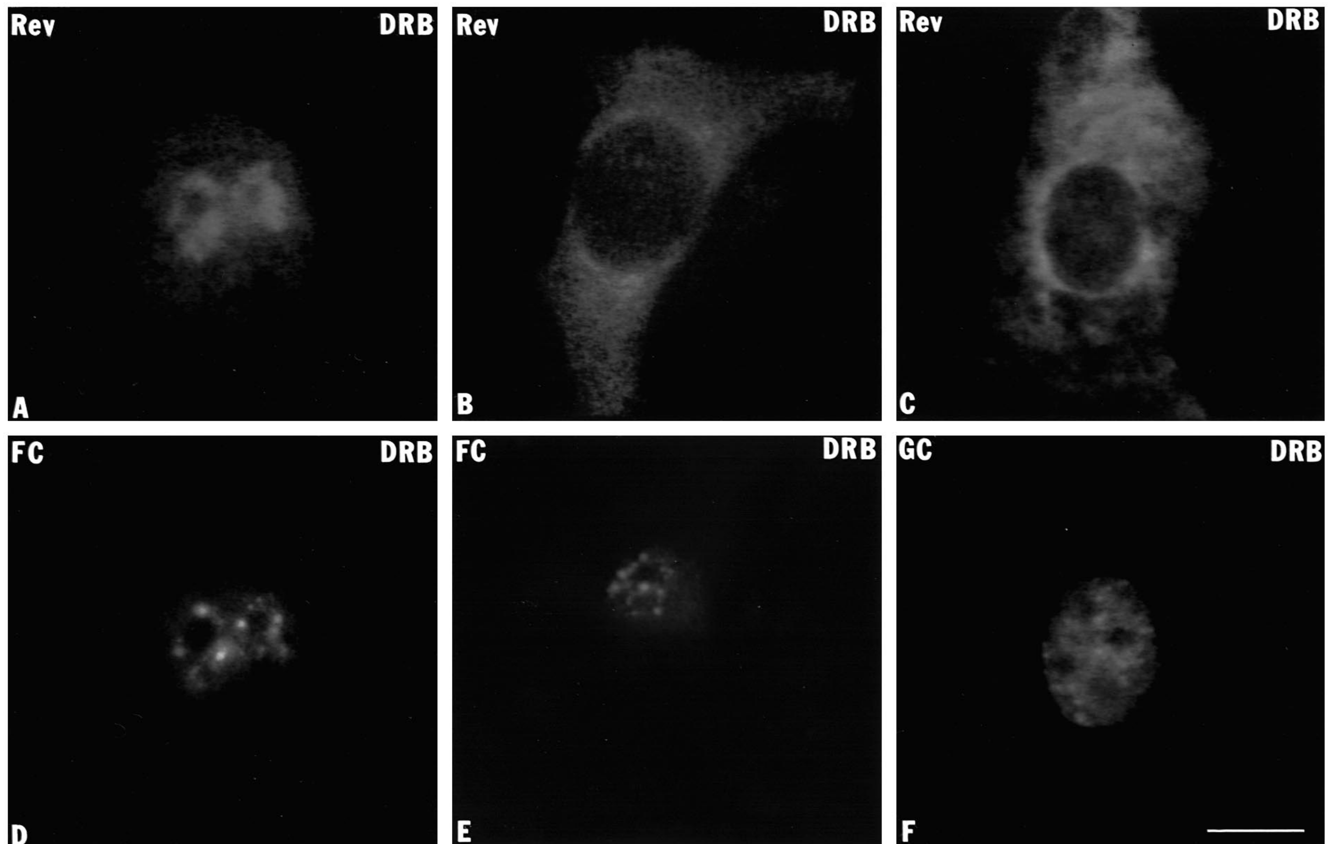

Fig. 9. Effects of DRB on the subcellular distribution of the Rev protein in Rev-expressing transformed CMT3 cells. The Rev protein remains in nucleoli after 3 hours of DRB (50 μg/ml) treatment (A). The apparent dispersion of FCs in nucleolar body is visible by labeling with anti-FC marker antibody (D). After 4 hours of DRB treatment there is significant redistribution of the Rev protein to the cytoplasm (B,C). Labeling with specific anti-FC (E) and anti-GC (F) marker antibodies reveals a relatively high level of nucleolar dispersion. Bar, 10 μm.

the Rev protein was still present in the dispersed nucleolar body after 6 hours of DRB treatment. Thus, the Rev protein appeared to remain in nucleoli under conditions where only RNA pol II is inhibited and before the secondary effects of DRB are seen (see below).

To confirm the above findings, we examined the effects of a second inhibitor, α-amanitin, which at low concentrations selectively inhibits transcription by RNA pol II and, at higher concentrations, RNA pol III, but does not block pre-rRNA synthesis (Roeder, 1976). Rev-expressing stable cells were incubated for 3 hours in a culture medium containing α-amanitin (10 μg/ml). The nucleolar location of Rev was not affected and nucleoli did not show any morphological alterations (Fig. 10A) compared with the untreated control. The distribution of fibrillarin (Fig. 10D) and protein B23 (data not shown) in nucleoli also appeared to be normal. However, when Rev-expressing stable cells were treated for 5 hours with a concentration of α-amanitin of 30 μg/ml which also blocks RNA pol III transcription, the Rev protein accumulated predominantly in the cytoplasm (Fig. 10B,C). The nucleoli of these cells exhibited a fragmented pattern of fibrillarin labeling that was localized in dispersed DFCs (Fig. 10E). Protein B23 as visualized by the anti-B23 mAb was mainly redistributed to the nucleoplasm but a portion was still present in round-shaped nucleolar bodies (Fig. 10F). A weak cytoplasmic signal for B23 after the high dose α-amanitin treatment was detectable only using the polyclonal anti-B23 antibody (data not shown). In summary, these data showed that the migration of the Rev protein to the cytoplasm was dependent on the transcriptional activity of RNA pol I and on the nucleolar structural integrity. Although transcription of rRNA genes continues during DRB treatment, the nucleoli become fragmented and lose their GCs, probably by degradation of RNA due to a deficiency in ribosomal proteins (Granick, 1975a,b; Scheer et al., 1984). This structural nucleolar remnant does not appear to be capable of retaining the Rev protein. With α-amanitin at high doses and long incubation times there appear to be secondary effects that mimic those seen when RNA pol I transcription is blocked by AMD.

The Rev protein redistribution to nucleoli during recovery from AMD treatment

Having shown that the Rev protein can migrate from the nucleus to the cytoplasm additional experiments were done to confirm the ability of the Rev protein to reaccumulate in nucleoli. The transiently transfected COS-7 cells were treated with a high dose of AMD (0.5 μg/ml) for 1 hour to ensure that in all class 1 cells the Rev protein had migrated to the cytoplasm. After extensive washing the cells were maintained in the presence of cycloheximide (20 μg/ml) to block protein synthesis de novo. In time course experiments the Rev protein was detectable in nucleoli after 3.5 hours of recovery (Fig.

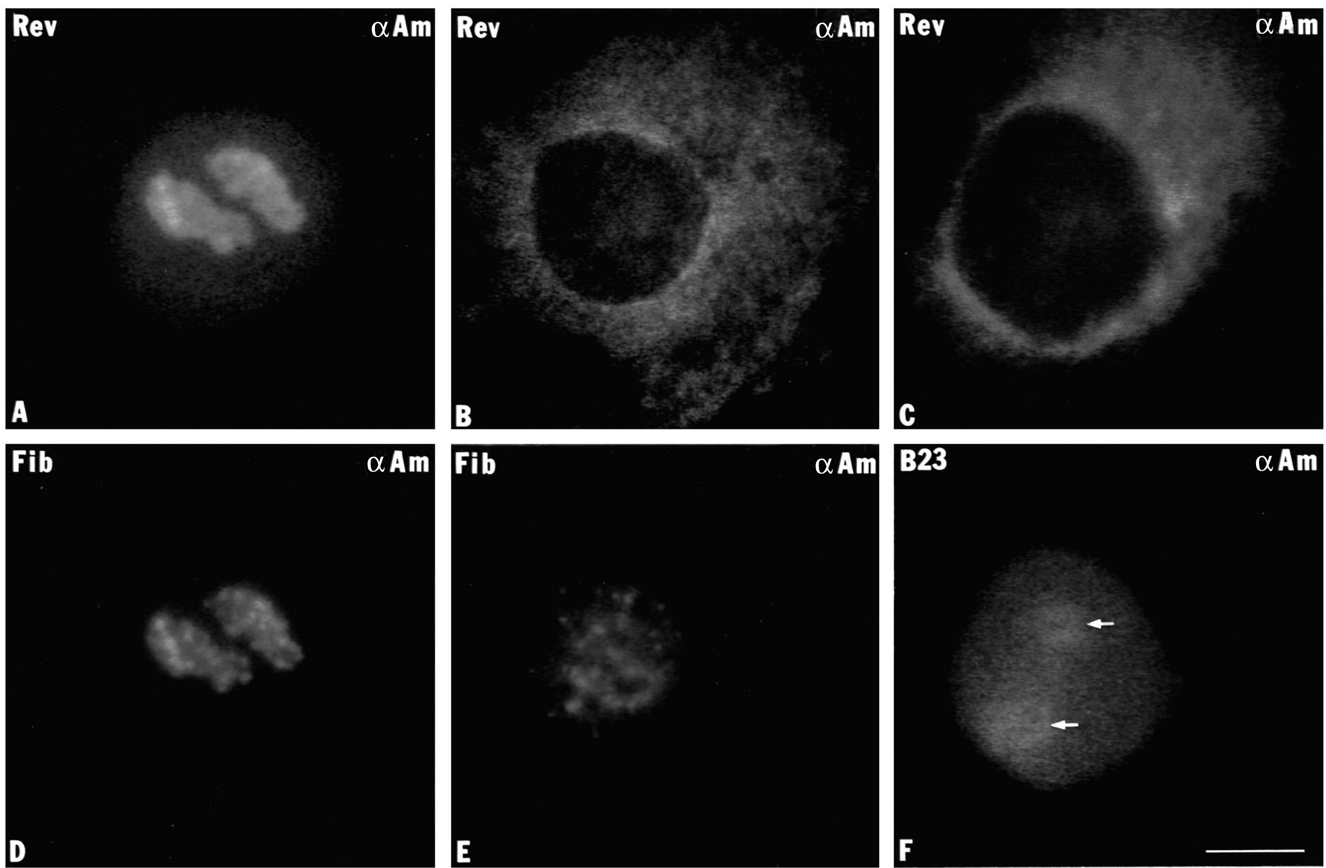

Fig. 10. Effects of α-amanitin on the subcellular distribution of the Rev protein in Rev-expressing transformed CMT3 cells. After α-amanitin treatment (10 μg/ml) for 3 hours the Rev protein remains in nucleoli (A), which exhibit essentially normal localization of fibrillarin (D). α-Amanitin treatment at a higher dose (30 μg/ml) and for a longer time (5 hours) causes translocation of the Rev protein from nucleoli and the nucleoplasm to the cytoplasm (B,C), with nucleoli showing a dispersed pattern of fibrillarin localization (E). Under the latter conditions protein B23 is mainly redistributed to the nucleoplasm with some remaining in fragmented nucleoli (F, arrows) as visualized by anti-B23 mAb. Bar, 10 μm.

11A, arrows). The time of recovery was the same in the absence of cycloheximide, suggesting that there was normally a stable steady-state pool of Rev protein in the cytoplasm. In stable Rev-expressing cell line under the same experimental conditions a longer time (5 hours) was required before Rev reappeared in nucleoli (Fig. 11B,C). The difference was possibly caused by a lower level of Rev protein expressed in these cells. Most nucleoli with reaccumulated Rev protein still exhibited a round shape and a segregated pattern of fibrillarin localization (Fig. 11D, arrows). The Rev protein was located predominantly in the peripheral part of the segregated nucleoli (Fig. 11A,B, arrows), where it colocalized with protein B23 (Fig. 11E, arrows). A significant proportion of the cell population exhibited only a slightly altered pattern of nucleolar structure with round shapes and a nearly complete nucleolar distribution of the Rev protein (Fig. 11C) which was coincident with protein B23 (Fig. 11F).

DISCUSSION

The current studies indicate that the Rev protein is predominantly localized to the nucleoli of cells expressing moderate amounts of the protein under conditions that maintain normal nucleolar structure and function. However, when preribosomal RNA transcription is inhibited or high levels of Rev accumulate the protein is not strictly nucleolar in location. The latter condition suggests that nucleoli contain a finite number of sites for accumulation and when these sites are saturated the Rev protein spills over into the nucleoplasm. Thus, nucleolar structure and function as well as the level of Rev expression strongly influence the subcellular location of the Rev protein.

The transiently transfected COS-7 cells provided evidence that the first site of Rev accumulation in the cell is the nucleolus. At short times (up to 3 hours) after transfection the Rev protein was predominantly in the nucleoli with only background levels of fluorescent labeling in the surrounding nucleoplasm. At later times after transfection there was a gradual increase in the number of cells accumulating Rev in the nucleoplasm, further supporting the idea that the nucleolus is the preferred site of Rev accumulation. The most likely route the Rev protein follows to the nucleolus is through the nucleoplasm by a two-step process (Girard et al., 1994). In this model there is receptor-mediated transport across the nuclear pore complex via nuclear localization sequences (NLSs), first to the nucleoplasm and then accumulation within the nucleolus via additional interactions with other macromolecules already present in the nucleolus. Our data suggest that most, if not all,

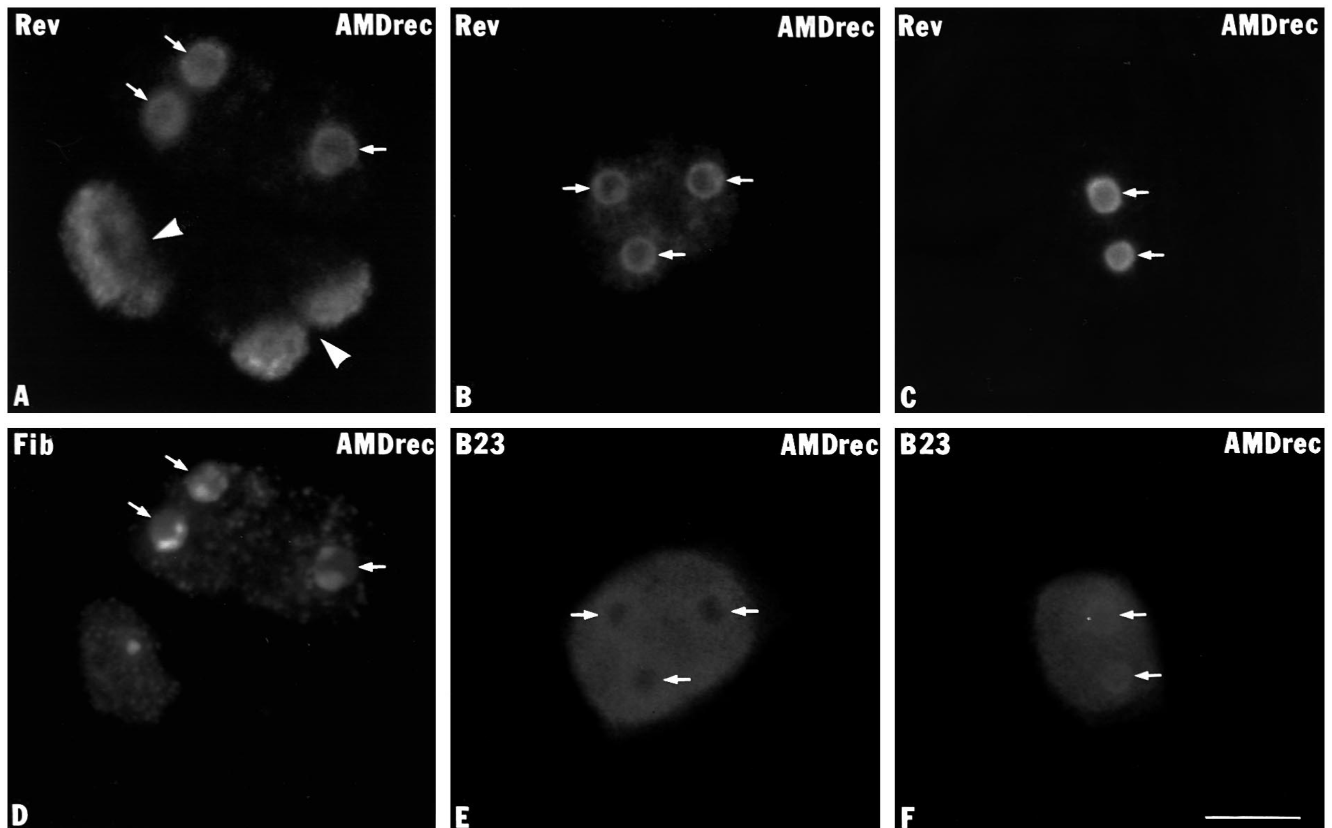

Fig. 11. Recovery of Rev-expressing cells after AMD treatment. Transiently transfected COS-7 (A) or transformed CMT3 (B,C) cells were treated with AMD (0.5 μg/ml) for 1 hour and then allowed to recover in the presence of cycloheximide (20 μg/ml) after removal of AMD from the medium. After 3.5 hours of recovery the Rev protein is detectable in the round-shaped nucleoli of class 1 cells (A, arrows) which exhibit a segregated pattern of fibrillarin localization (D, arrows). Class 2 cells (arrowheads) were insensitive to AMD induced translocation (A). In the stable Rev-expressing cells after 6 hours of recovery the Rev protein is localized predominantly in the peripheral part of segregated nucleoli (B, C, arrows), where it is colocalized with the segregated distribution of protein B23 (E, F, arrows). Bar, 10 μm.

of the Rev protein is primarily targeted to the nucleolus after entry into the nucleus.

At least two lines of evidence indicate that an actively transcribing nucleolus is necessary for nucleolar location of the Rev protein. First, the Rev protein is located in two of the three fundamental nucleolar components, the DFC and GC, which are composed of the products of rDNA transcription, ribosomal proteins and proteins involved in preribosomal particle maturation. Secondly, studies using RNA pol inhibitors provide evidence for the essential role of rDNA transcription. Selective inhibition of RNA pol I caused rapid release of the Rev protein to the nucleoplasm and ultimately to the cytoplasm. The effects of AMD appear to be reversible; i.e. removal of AMD from the medium allowed Rev to slowly return to the nucleolus. Furthermore, nucleolar reaccumulation did not require new protein synthesis. Thus, the Rev protein apparently can move from the nucleolus to the cytoplasm and from the cytoplasm back to the nucleolus without being resynthesized as suggested by recent studies (Meyer and Malim, 1994; Richard et al., 1994). However, reaccumulation in the nucleolus apparently requires transcription of preribosomal RNA and the restoration of a generally normal nucleolus.

Curiously, cells expressing apparently very high levels of Rev did not respond to AMD treatment. This is best explained by the high affinity of Rev for nucleolar components which are drawn out of the nucleolus by overwhelming amounts of Rev. In this case Rev is concentrated at the periphery of nuclei, as if it is waiting to be exported to the cytoplasm. A similar distribution of Rev was observed by Kalland et al. (1994). Overproduction of the Rev protein may saturate the transport system and prevent translocation into the cytoplasm.

In Rev-expressing cells treated with α-amanitin at concentrations that only inhibit RNA pol II there was no release of the Rev protein from the nucleolus. Furthermore, treatment with DRB at concentrations which inhibit RNA pol II, but not RNA pol I did not cause release of the Rev protein. In the latter case there was minimal disruption of the nucleolus. Thus, RNA pol I, but not RNA pol II transcription appears to be essential for the nucleolar location of the Rev protein.

Recently, Meyer and Malim (1994) demonstrated that the Rev protein shuttles between the nucleus and cytoplasm. They also concluded that the accumulation of the Rev protein in cell nuclei is dependent on the activity of RNA pol II, because the inhibition of RNA pol II alone by DRB or by high doses of AMD led to the relocalization of wild-type Rev to the cytoplasm. However, our data showed that on the one hand, selective inhibition of RNA pol I led to translocation of the Rev protein to the cytoplasm. On the other hand, selective inhibition of RNA pol II by α-amanitin or DRB did not cause the translocation. The discrepancies between our data and those of

Meyer and Malim (1994) could be due to a number of factors. First, different cell types may respond differently to the agents used. Second, the response to the drugs may be dependent on the level of expression of the Rev protein. Third, the effects are time dependent and must be optimized for each cell type. Finally, it is important to distinguish between direct and indirect effects of the drugs used. For example, DRB selectively suppresses RNA pol II transcription and induces dispersal of compact nucleoli into extended beaded strands termed nucleolar necklaces (Granick, 1975b; Scheer et al., 1984). Although RNA pol I transcription is not affected, DRB causes RNA degradation in large preribosomal subunits (Granick, 1975a,b). The translocation of Rev is not seen at early times after DRB treatment because the rDNA transcription units remain intact. However, with longer times of treatment the effects of RNA pol II inhibition are seen because ribosomal and other proteins are not available for ribosome assembly. Similarly, selective inhibition of RNA pol II by α-amanitin induces the fragmentation of nucleoli accompanied by aggregation of ribonucleoprotein components, followed by nucleolar disaggregation (Sinclair and Brasch, 1978).

A major question raised by the presence of Rev in the nucleolus is what is the nucleolar receptor. The high affinity of nucleolar protein B23 for Rev has clearly been demonstrated (Fankhauser et al., 1991) and the current work indicates that Rev and B23 localize in the same components of nucleoli. Furthermore, Rev and B23 are simultaneously released from nucleoli in response to treatment by various drugs (see above). Thus, the available evidence suggests that the two proteins are associated with each other in cells. However, the current studies did not allow us to determine what proportion of the Rev protein is bound to B23. It has been shown that Rev is able to bind nonspecifically to RNA molecules with approximately 20-fold lower affinity than for the RRE (Daly et al., 1989; Heaphy et al., 1990). Because of the abundance of RNA in the nucleolus it is highly probable that a portion of Rev is bound to nucleolar RNA. Thus protein B23 may not be the sole receptor for Rev in the nucleolus.

The final question is whether there is any functional role for the nucleolar location of Rev. Deletion or extensive mutation of the NOS in Rev blocks nucleolar accumulation and destroys its ability to regulate HIV-1 mRNA expression (Malim et al., 1989; Cochrane et al., 1990). However, the NOS of the Rev protein binds the RRE of the viral message as well as directing the protein to the nucleolus, indicating a dual function for this segment of the protein. Because of this duality in function, it could be argued that nucleolar localization is fortuitous and has no real function. However, the current studies reinforce the strong tendency of the Rev protein to preferentially accumulate in the nucleolus. Furthermore, the movement of the Rev protein into the cytoplasm is closely coupled with nucleolar function and structure. Therefore, the nucleolus could serve as a buffer zone or temporary storage area for Rev to be available for binding to viral mRNA. It is possible that the later stages of HIV-1 infection could induce a decrease in nucleolar output which in turn may release the Rev protein into the nucleoplasm. This may provide the proper timing for the regulation of mRNA splicing during the switch to production of structural proteins. Better systems for assay of the role of the nucleolus in HIV-1 function will have to be developed before this question can be fully answered.

The authors thank Drs Julio Herrera and Dunrui Wang for helpful discussions; Drs Danièle Hernandez-Verdun, Robert L. Ochs and Pui K. Chan for providing antisera; and Mrs Amy Baumann for assistance in plasmid preparation. This work was supported by NIH grants AI34277 (to M.O.) and AI25721 (to M.L.H.).

REFERENCES

Adachi, Y., Copeland, T. D., Hatanaka, M. and Oroszlan, S. (1993). Nucleolar targeting signal of Rex protein of human T-cell leukemia virus type I specifically binds to nucleolar shuttle protein B-23. *J. Biol. Chem.* **268**, 13930-13934.

Arrigo, S. J. and Chen, I. S. Y. (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.

Bray, M., Prasad, S., Dubay, J. W., Hunter, E., Jeang, K.-T., Rekosh, D. and Hammarskjöld, M.-L. (1994). A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proc. Nat. Acad. Sci. USA* **91**, 1256-1260.

Campbell, L. H., Borg, K. T., Haines, J. K., Moon, R. T., Schoenberg, D. R. and Arrigo, S. J. (1994). Human immunodeficiency virus type 1 is required in vivo for binding of poly(A)-binding protein to Rev-dependent RNAs. *J. Virol.* **68**, 5433-5438.

Chang, D. D. and Sharp, P. A. (1989). Regulation by HIV Rev depends upon recognition of splice sites. *Cell* **59**, 789-795.

Cochrane, A. W., Perkins, A. and Rosen. (1990). Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. *J. Virol.* **64**, 881-885.

Cullen, B. R. (1987). Use of eukaryotic expression technology in the functional analysis of cloned genes. *Meth. Enzymol.* **152**, 684-703.

Cullen, B. R., Hauber, J., Campbell, K., Sodroski, G., Haseltine, W. A. and Rosen, C. A. (1988). Subcellular localization of the human immunodeficiency virus trans-acting art gene product. *J. Virol.* **62**, 2498-2501.

DíAgostino, D. M., Felber, B. K., Harrison, J. E. and Pavlakis, G. N. (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.

Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E. and Rusche, J. R. (1989). Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element in vitro. *Nature* **342**, 816-819.

Dundr, M. and Raška, I. (1993). Nonisotopic ultrastructural mapping of transcription sites within the nucleolus. *Exp. Cell Res.* **208**, 275-281.

Emerman, M., Vazeux, R. and Peden, K. (1989). The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* **57**, 1155-1165.

Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P. and Laemmli, U. K. (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.

Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. and Pavlakis, G. N. (1989). Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc. Nat. Acad. Sci. USA* **86**, 1495-1499.

Fischer, U., Meyer, S., Teufel, M., Heckel, C., Lührmann, R. and Rautmann, G. (1994). Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J.* **13**, 4105-4112.

Gerard, R. D. and Gluzman, Y. (1985). New host cell system for regulated simian virus 40 DNA replication. *Mol. Cell. Biol.* **5**, 3231-3240.

Girard, J.-P., Bagni, C., Caizergues-Ferrer, M., Amalric, F. and Lapeyre, B. (1994). Identification of a segment of the small nucleolar ribonucleoprotein-associated protein GAR1 that is sufficient for nucleolar accumulation. *J. Biol. Chem.* **269**, 18499-18506.

Granick, D. (1975a). Nucleolar necklaces in chick embryo fibroblast cells. I. Formation of necklaces by dichlororibobenzimidazole and other adenosine analogues that decrease RNA synthesis and degrade preribosomes. *J. Cell Biol.* **65**, 389-417.

Granick, D. (1975b). Nucleolar necklaces in chick fibroblast cells. II. Microscope observations of the effect of adenosine analogues on nucleolar necklace formation. *J. Cell Biol.* **65**, 418-427.

Hadzopoulou-Cladaras, M., Felber, B. K., Cladaras, C., Athanassopoulos,

A., Tse, A. and Pavlakis, G. N. (1989). The Rev (Trs/Art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. *J. Virol.* **63**, 1265-1274.

Hammarskjöld, M.-L., Heimer, J., Hammarskjöld, B., Sangwan, I., Albert, L. and Rekosh, D. (1989). Regulation of human immunodeficiency virus env expression by the rev gene product. *J. Virol.* **63**, 1959-1966.

Hammarskjöld, M.-L., Li, H., Rekosh, D. and Prasad, S. (1994). Human immunodeficiency virus env expression becomes Rev-independent if the env region is not defined as an intron. *J. Virol.* **68**, 951-958.

Heaphy, S., Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Karn, J., Lowe, A. D., Singh, M. and Skinner, M. A. (1990). HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. *Cell* **60**, 685-693.

Hendzel, M. J. and Bazett-Jones, D. P. (1995). RNA polymerase II transcription and the functional organization of the mammalian cell nucleus. *Chromosoma* **103**, 509-516.

Huang, S., Deerinck, T. J., Ellisman, M. H. and Spector, D. L. (1994). In vivo analysis of the stability and transport of nuclear poly(A)$^+$ RNA. *J. Cell Biol.* **126**, 877-899.

Kalland, K. H., Szilvay, A. M., Langhoff, E. and Haukenes, G. (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.

Kjems, J., Frankel, A. D. and Sharp, P. A. (1991). Specific regulation of mRNA splicing in vitro by a peptide from HIV-1 Rev. *Cell* **67**, 169-178.

Kjems, J. and Sharp, P. A. (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.

Kubota, S., Siomi, H., Satoh, T., Endo, S.-I., Maki, M. and Hatanaka, M. (1989). Effects of chimeric mutants of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex on nucleolar targeting signal. *J. Virol.* **65**, 2452-2456.

Lewis, N., Williams, J., Rekosh, D. and Hammarskjöld, M.-L. (1990). Identification of a cis-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 rev and human T-cell leukemia virus types I and II rex proteins. *J. Virol.* **64**, 1690-1697.

Lu, X., Heimer, J., Rekosh, D. and Hammarskjöld, M.-L. (1990). U1 small nuclear RNA plays a direct role in the formation of a rev-related human immunodeficiency virus env mRNA that remains unspliced. *Proc. Nat. Acad. Sci. USA* **87**, 7598-7602.

Malim, M. H., Böhnlein, S., Hauber, J. and Cullen, B. R. (1989). Functional dissection of the HIV-1 Rev trans-activator derivation of a trans-dominant repressor of Rev function. *Cell* **58**, 205-214.

Malim, M. H. and Cullen, B. R. (1991). HIV-1 structural gene expression requires the binding of multiple Rev monomers to viral RRE: implications for HIV-1 latency. *Cell* **65**, 241-248.

Malim, M. H. and Cullen, B. R. (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.

Meyer, B. E. and Malim, M. H. (1994). The HIV-1 Rev trans-acrivator shuttles between the nucleus and the cytoplasm. *Genes Dev.* **8**, 1538-1547.

Olsen, H. S., Cochrane, A. W., Dillon, P. J., Nalin, C. M. and Rosen, C. A. (1990). Interaction of the human immunodeficiency virus 1 Rev protein with a structured region in env mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. *Genes Dev.* **4**, 1357-1364.

Perry, R. P. and Kelley, D. E. (1968). Persistent synthesis of 5S RNA when production of 28S and 18S ribosomal DNA is inhibited by low doses of actinomycin D. *J. Cell. Physiol.* **72**, 235-246.

Perry, R. P. and Kelley, D. E. (1970). Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. *J. Cell. Physiol.* **76**, 127-139.

Richard, N., Iacampo, S. and Cochrane, A. (1994). HIV-1 Rev is capable of shuttling between the nucleus and cytoplasm. *Virology* **204**, 123-131.

Roeder, R. G. (1976). Eukaryotic nuclear RNA polymerases. In *RNA Polymerase* (ed. R. Losick and M. Chamberlin), pp. 285-329. Cold Spring Harbor Laboratory Press, New York.

Rosen, C. A., Terwilliger, E., Dayton, A., Sodroski, J. G. and Haseltine, W. A. (1988). Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. *Proc. Nat. Acad. Sci. USA* **85**, 2071-2075.

Scheer, U., Hügle, B., Hazan, R. and Rose, K. M. (1984). Drug-induced dispersal of transcribed rRNA genes and trascriptional products: Immunolocalization and silver staining of different nucleolar components in rat cells treated with 5, 6-dichloro-β-D-ribofuranosylbenzimidazole. *J. Cell Biol.* **99**, 672-679.

Scheer, U. and Benavente, R. (1990). Functional and dynamic aspects of the mammalian nucleolus. *BioEssays* **12**, 14-21.

Sinclair, G. D. and Brasch, K. (1978). The reversible action of α-amanitin on nuclear structure and molecular composition. *Exp. Cell Res.* **111**, 1-14.

Slot, J. W. and Geuze, H. J. (1984). Gold markers for single and double immunolabelling of ultrathin cryosections. In *Immunolabelling for Electron Microscopy* (ed. J. M. Polak and I. M. Varndell), pp. 129-142. Elsevier Science Publishing, Amsterdam.

Smith, A. J., Cho, M. I., Hammarskjöld, M.-L., and Rekosh, D. (1990). Human immunodeficiency virus type 1 Pr55gag and Pr160gag-pol expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. *J. Virol.* **64**, 2743-2750.

Sugden, B., Marsh, K., and Yates, J. (1985). A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. *Mol. Cell. Biol.* **5**, 410-413.

Tiley, L. S., Malim, M. H., Tewary, H. K., Stockley, P. G. and Cullen, B. R. (1992). Identification of a high-affinity RNA-binding site for the human immunodeficiency virus type 1 Rev protein. *Proc. Nat. Acad. Sci. USA* **89**, 758-762.

Umekawa, H., Chang, J. H., Correia, J. J., Wang, D., Wingfield, P. T. and Olson, M. O. J. (1993). Nucleolar protein B23: bacterial expression, purification, oligomerization and secondary structures of two isoforms. *Cell. Mol. Biol. Res.* **39**, 635-645.

Zapp, M. L., Hope, T. J., Parslow, T. G. and Green, M. R. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. *Proc. Nat. Acad. Sci. USA* **88**, 7734-7738.

(Received 21 November 1994 - Accepted 17 May 1995)