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Location of the HIV-1 Rev protein during mitosis: inactivation of the nuclear export signal alters the pathway for postmitotic reentry into nucleoli

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SUMMARY

The HIV-1 Rev protein localizes predominantly to the nucleolus of HIV-1-infected or Rev-expressing cells. The subcellular location of Rev during mitotic nucleolar disintegration was examined at various stages of mitosis in synchronized Rev-expressing CMT3 cells. During early prophase Rev was predominantly located in disintegrating nucleoli and began to accumulate at the peripheral regions of chromosomes in late prophase, eventually distributing uniformly on all chromosomes in prometaphase. In anaphase Rev remained associated with the perichromosomal regions, but significant amounts of Rev were also seen in numerous nucleolus-derived foci. The movement of Rev from disintegrating nucleoli to perichromosomal regions and foci was similar to that of nonribosomal nucleolar proteins, including fibrillarin, nucleolin, protein B23 and p52 of the granular component. During telophase Rev remained associated with perichromosomal regions and mitotic foci until the nuclear envelope started to reform. When nuclear envelope formation was complete in late telophase, nonribosomal nucleolar proteins were present in prenucleolar bodies (PNBs) which were eventually incorporated into nucleoli; at the same time, Rev was excluded from nuclei. In contrast, a *trans*-dominant negative Rev protein containing an inactive nuclear export signal reentered nuclei by the nonribosomal nucleolar protein pathway in late telophase, associating with PNBs and reformed nucleoli. Rev protein reentry into postmitotic nuclei was delayed until early G_1 phase, but before the arrival of ribosomal protein S6. Thus, Rev behaves like a nonribosomal nucleolar protein through mitosis until early telophase; however, its nuclear reentry seems to require reestablishment of both a nuclear import system and active nucleoli.

Key words: HIV-1 Rev protein, Mitosis, Nuclear export signal, Nucleologenesis, Prenucleolar bodies

INTRODUCTION

The HIV-1 Rev protein is a nuclear *trans*-activator essential for the expression of viral structural proteins from unspliced (gag/pol) and incompletely spliced (env) HIV-1 mRNAs in late phases of infection (Emerman et al., 1989; Felber et al., 1989; Hammarskjöld et al., 1989; Malim et al., 1989). The Rev protein regulates the transport of viral mRNAs from the nucleus to the cytoplasm through the direct interaction of the basic arginine-rich domain with a 234 nt Rev response element (RRE) present in the *env* intron region of the HIV-1 mRNA (Rosen et al., 1988; Daly et al., 1989; Hadzopoulou-Cladaras et al., 1989). This domain of the Rev protein also acts as a nucleolar targeting signal (NOS); nuclear targeting of the Rev protein is essential for its function (Kubota et al., 1989; Malim et al., 1989; Cochrane et al., 1990). The sequences flanking this domain are necessary for Rev protein multimerization (Malim and Cullen, 1991). Accumulated evidence suggests that the Rev protein directly promotes export of RRE-containing pre-mRNAs (Hammarskjöld et al., 1989, 1994; Malim and Cullen, 1993; Fischer et al., 1994). A second leucine-rich domain, called the activation domain (~10 amino acids) located close to the C terminus contains the nuclear export signal (NES). Through it the Rev protein is believed to interact with one or more cellular factors to promote RNA export (Bogerd et al., 1995; Fischer et al., 1995; Fritz et al., 1995; Stutz et al., 1995; Wen et al., 1995; reviewed by Gerace, 1995; Görlich and Mattaj, 1996).

The Rev protein is predominantly located in the nucleoli of expressing cells (Kubota et al., 1989; Malim et al., 1989; Cochrane et al., 1990). Although nucleolar localization is not an absolute requirement for Rev function (McDonald et al., 1992), recent studies (Kubota et al., 1996) suggest that the nucleolus is a Rev storage site that prevents Rev degradation and maintains optimal levels of the protein for its function (Pomerantz et al., 1992). The Rev protein may also be taking advantage of the import system that transports large quantities of ribosomal proteins to the nucleolus, with the latter location being a temporary stop on the way to the nucleoplasm where the viral mRNA is synthesized. The nucleolar location of Rev requires the nucleolus to be actively engaged in preribosomal RNA transcription (Dundr et al., 1995). A candidate host cell

factor for interacting with the Rev protein was identified as nucleolar shuttling protein B23 (Fankhauser et al., 1991). The latter protein and Rev colocalize in the nucleoli of Rev-expressing cells and show similar redistribution patterns during recovery from blocked ribosomal RNA (rRNA) transcription (Dundr et al., 1995).

During mitosis cell nuclei undergo extensive structural reorganization. The nucleoli disintegrate during early prophase (Goessens, 1984) and the nucleolar proteins differentially redistribute within the dividing cells. Some nucleolar proteins remain associated with the nucleolar organizer regions (NORs), others associate with the periphery of chromosomes where they remain until late telophase and some uniformly disperse throughout the cytoplasm (Sommerville, 1986). Among the nucleolar proteins stably associated with the NORs during mitosis are those that belong to the RNA polymerase (pol) I transcription complex: RNA pol I (Scheer and Rose, 1984; Haaf et al., 1988; Gilbert et al., 1995), transcription factor UBF (Chan et al., 1991; Rendon et al., 1992; Roussel et al., 1993), DNA topoisomerase I (Guldner et al., 1986) and the NOR protein pp135 (Pfeifle et al., 1986). Those nucleolar proteins located in the chromosomal peripheral regions include perichromonucleolin (Shi et al., 1987), proliferation-associated Ki-67 antigen (Verheijen et al., 1989), protein B23/No38 (Ochs et al., 1983; Schmidt-Zachmann et al., 1987), fibrillarin (Yasuda and Maul, 1990; Azum-Gélade et al., 1994; Weisenberger and Scheer, 1995), nucleolin (Weisenberger and Scheer, 1995), p52, p68 and p103 (Gautier et al., 1992), ribocharin (Hügle et al., 1985a) and ribosomal protein S1 (Hügle et al., 1985b). The redistribution throughout the cytoplasm during prophase to telophase has been reported for ribosomal protein S6 (Jiménez-García et al., 1994) and nucleolar phosphoprotein p130 (Pai et al., 1995).

During nucleologenesis in early telophase, specific nucleolar proteins begin to associate with prenucleolar bodies (PNBs) in the newly formed daughter nuclei. In late telophase when RNA pol I transcription is reinitiated the PNBs migrate towards the NORs where they fuse and become the dense fibrillar components of the nucleolus (Scheer et al., 1993; Jiménez-García et al., 1994; Scheer and Weisenberger, 1994).

Because there is little or no transcriptional activity during mitosis until telophase, the early stages of mitosis provide a model system for in vivo analyses of the relationship between the Rev protein and other nucleolar proteins when nucleoli are dispersing. Conversely, the segment of the cell cycle from late telophase to early G_1 phase when RNA synthesis begins again, provides an opportunity to study the relationship between the Rev protein and other nucleolar proteins during nuclear and nucleolar reassembly.

The current studies were undertaken to determine the location of the Rev protein when the nucleolus disintegrates during mitosis and the path it follows to reenter the reforming nucleolus. It was found that the wild-type Rev, a *trans*-dominant negative Rev (TD Rev) and several nonribosomal nucleolar proteins associate with the peripheral regions of chromosomes during prometaphase and are found in nucleolus-derived foci in anaphase and telophase. The nonribosomal nucleolar proteins and the TD Rev protein associate with prenucleolar bodies and reforming nucleoli in late telophase. In contrast, the entry of wild-type Rev into nuclei and nucleoli is delayed until early G_1 phase and does not occur via the prenucleolar bodies. Thus, Rev does not assemble into reforming nucleoli via the pathway typical of most nonribosomal nucleolar proteins.

MATERIALS AND METHODS

Cell cultures

The CMT3 monkey cell line (Gerard and Gluzman, 1985) was grown on poly-L-lysine-coated glass coverslips in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% FCS (Gibco BRL) in 5% CO_2 atmosphere. The A5.9 cell line was described previously (Dundr et al., 1995). This cell line was obtained by transfection of CMT3 cells with the plasmid pCMVrev together with pHyg, a plasmid that confers hygromycin resistance. The B4.14 cell line was obtained after transfection of CMT3 cells with pCMVrev, pCMVgag/pol-RRE-r and pHyg. These cells express the HIV-1 gag/pol protein in a Rev-dependent manner (Smith et al., 1990). The 2AB7 cell line was generated after cotransfection of CMT3 cells with pCMVrevD78-79 and pHyg. The plasmid pCMVrevD78-79 expresses a *trans*-dominant negative HIV-1 Rev protein (TD Rev) which has a deletion of two amino acids (aa 78 and 79) in the nuclear export signal of Rev. This plasmid was generated from pCMVrev by site-specific mutagenesis using PCR. The cells were synchronized at the G_1/S transition by a double thymidine block with 2.5 mM thymidine (Bootsma et al., 1964). The cells were then released for approximately 8 hours to proceed through mitosis.

To determine the nucleus-to-cytoplasm shuttling characteristics of the TD Rev protein the TD Rev-expressing CMT3 cells were treated with 0.5 μg/ml of actinomycin D in time course experiments.

Immunofluorescence

Coverslips with attached cells were washed in PBS and fixed with 3% paraformaldehyde in PBS for 20 minutes at room temperature. After fixation, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes on ice and then washed extensively with 1% BSA in PBS. Immunofluorescence was performed as previously described (Dundr et al., 1995). In the case of double fluorescence labeling of the Rev protein and DNA, the cells were treated with propidium iodide (0.2 μg/ml) (Sigma) for 15 minutes with subsequent treatment with RNase A (10 μg/ml). After extensive washing, the secondary antibody conjugated with FITC was used for detection of the Rev protein. The Rev protein was detected using a monoclonal antibody (mAb) (Repligen), a rabbit polyclonal antibody (American Biotechnologies) or a polyclonal antibody against the C-terminal end of the protein. Fibrillarin was detected with human autoimmune serum S4 and ribosomal protein S6 with rabbit polyclonal antibody (kindly provided by Dr R. L. Ochs). Fibrillar centers were detected using the V11 autoimmune serum against the 62 kDa subunit of RNA polymerase I and a 52 kDa ribonucleoprotein from the granular components was visualized with G04 autoimmune serum (both kindly provided by Dr D. Hernandez-Verdun). Protein B23 was detected using a polyclonal antibody raised against rat recombinant protein B23.1 purified by the procedure described by Umekawa et al. (1993) and anti-B23 mAb (kindly provided Dr P. K. Chan). Nucleolin was labeled using a polyclonal antibody against the N-terminal end of nucleolin. A hybridoma cell line that secretes a mAb against splicing factor SC35 was obtained from the American Type Culture Collection (CRL-2031).

Fluorescence microscopy

The samples were examined using a laser scanning confocal microscope (Noran) with either a Nikon ×60/1.4 NA or ×100/1.3 NA objective. For double labeling, samples were subjected to an excitation wavelength of 488 nm (fluorescein) or 529 nm (Texas red, propidium iodide) from an argon-ion laser. The confocal images for each fluorochrome from the same confocal plane were recorded inde-

pendently and photographed using Kodak T-Max 400 film with a digital palette (Polaroid). In some cases, the images obtained with different fluorochromes were recorded independently, pseudocolored, superimposed and photographed on Kodak Gold 400 film.

RESULTS

Immunolocalization of the Rev protein during mitosis

We previously showed that during interphase the Rev protein is located predominantly in the dense fibrillar components (DFCs) and the granular components (GCs) of nucleoli of Rev-expressing cells (Dundr et al., 1995). Futhermore, the nucleolar location of Rev depends on continuing preribosomal RNA (pre-rRNA) transcription. Because pre-rRNA transcription is shut down as nucleoli disintegrate during mitosis, we examined the subcellular location of the Rev protein especially as related to its temporal and spatial association with nucleolar proteins as the cells proceeded through the mitotic cycle. This was done using a monoclonal antibody against the Rev protein to determine its location relative to DNA that was labeled with propidium iodide.

As the Rev-expressing CMT3 cells progressed from interphase to early prophase the Rev protein began to move out of disintegrating nucleoli and disperse among the newly-formed chromosomes (Fig. 1A,D). By late prophase most of the Rev protein had left the nucleoli to form a clear outline of the condensing chromosomes before breakdown of the nuclear envelope (Fig. 1B,E). During prometaphase the Rev protein completely surrounded the chromosomes (Fig. 1C,F). There were no detectable differences in the intensities of perichromosomal staining of the Rev protein among the NOR-bearing chromosomes and the other chromosomes. When the chromosomes lined up along the spindle microtubules to form the metaphase plate the perichromosomal labeling of Rev was clearly visible (Fig. 2A,D). At metaphase the Rev protein was essentially uniformly distributed over the periphery of chromosomes in the metaphase plate without significant cytoplasmic labeling (Fig. 2B,E). The inner part of the metaphase plate in which the centromeric regions of chromosomes were concentrated was weakly labeled. During anaphase the Rev protein followed the chromosomes and associated with the perichromosomal regions as they migrated to opposite poles; however, a significant amount of the Rev protein was also seen in numerous particles which we have termed ‘nucleolus-derived foci’ (NDF) (Fig. 2C,F,G,J, arrows). The NDF appeared to be randomly distributed over the cytoplasm with some of them close to the cell periphery but excluded from the mitotic spindle region. The number of the NDF during anaphase was typically about 25-50 per cell although some cells contained over 100. These were also highly variable in size.

During early telophase a significant portion of the Rev protein remained in close contact with peripheral regions of chromosomes and in foci (see below). When the nuclear envelope started to re-form around individual chromosomes

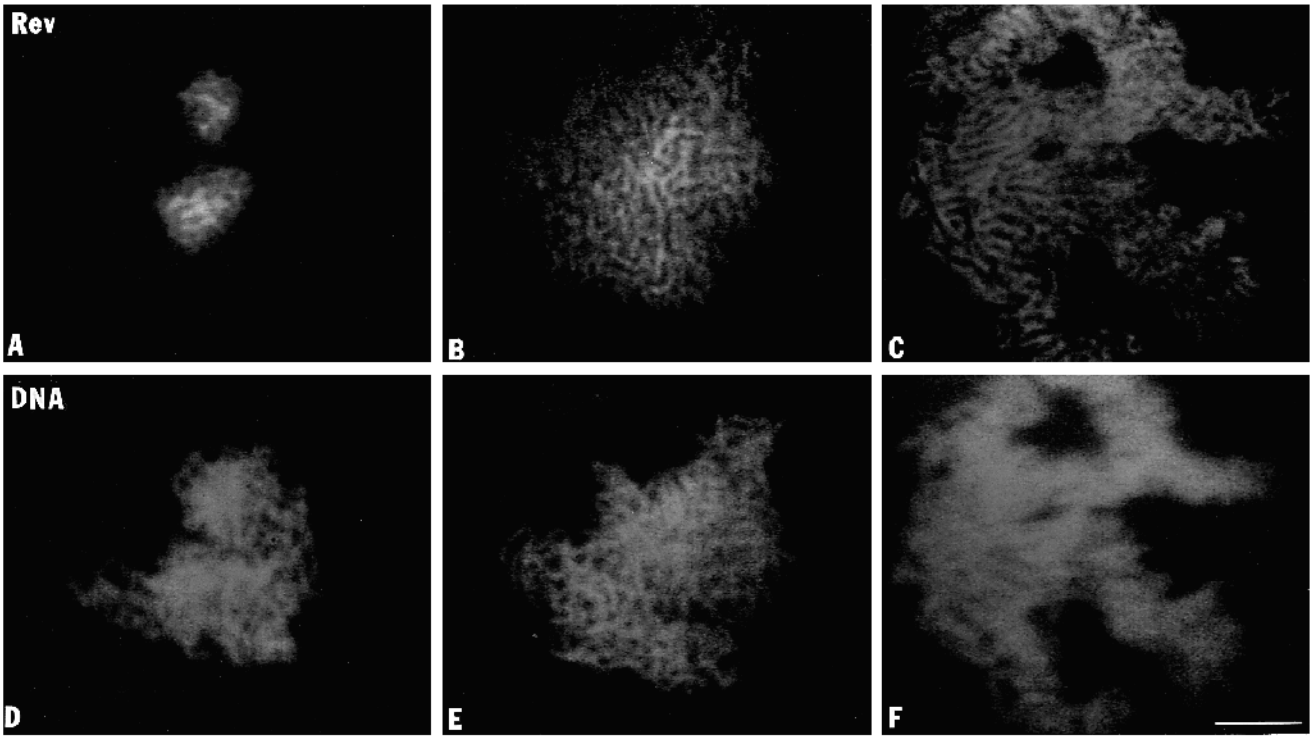

Fig. 1. Localization of the Rev protein and DNA in early phases of mitosis in Rev-expressing CMT3 cells. Cells from line A5.9 were synchronized as described in Materials and Methods and subjected to double immunofluorescence confocal microscopy. After release from double thymidine block, cells proceeding through mitosis were treated with anti-Rev antibody and fluorescein-labeled secondary antibody and with propidium iodide for DNA staining. In Figs 1 and 2 all panels are paired, with the upper panel showing anti-Rev staining and the lower panel indicating DNA staining in the same optical section. In early prophase the Rev protein is predominantly localized in disintegrating nucleoli (A,D). In late prophase the Rev protein is seen in the outlines of the condensed chromosomes (B,E). In prometaphase the Rev protein is present in the perichromosomal regions of all chromosomes (C,F). Bar, 10 μm.

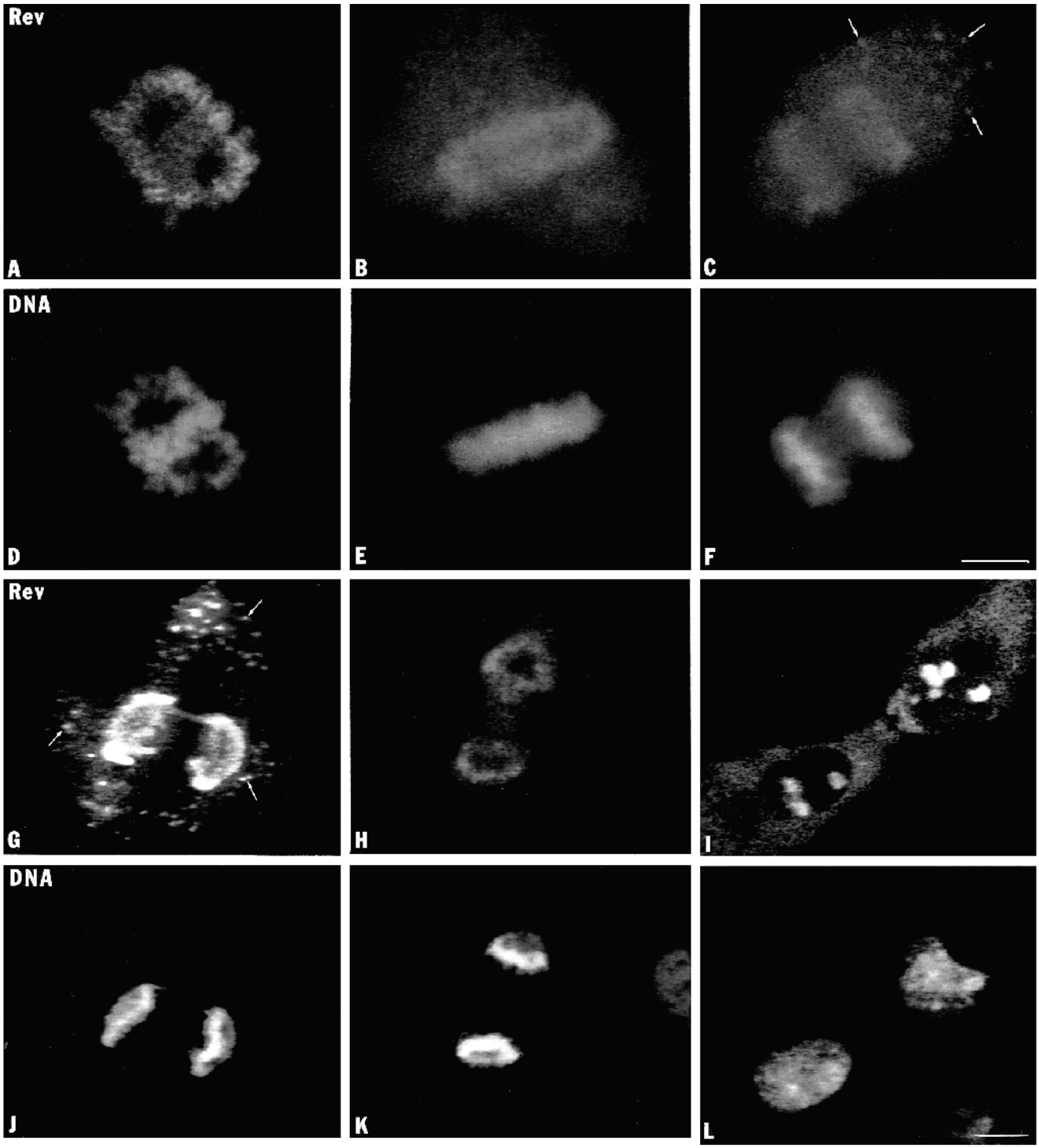

Fig. 2. Localization of the Rev protein and DNA in Rev-expressing CMT3 cells between prometaphase and early G_1 phase. The experimental conditions are the same as in Fig. 1. In late prometaphase Rev clearly decorates the periphery of chromosomes that are becoming oriented toward the metaphase plate (A,D). During metaphase the Rev protein is concentrated in the periphery of chromosomes in the metaphase plate with reduced staining at the equator (B,E). In early anaphase the Rev protein is localized in the perichromosomal region and in nucleolus-derived foci located outside the spindle region (C, arrows, F). In late anaphase the Rev protein is seen in the chromosomal periphery and in the nucleolus-derived foci (G, arrows, J). During early telophase the Rev protein is present in the periphery of decondensing chromosomes (H,K). In early G_1 phase the Rev protein is predominantly localized in reformed nucleoli and to lesser extent in the cytoplasm (I,L). Bars, 10 μm.

the Rev protein was still clearly visible in association with the chromosome arms predominantly in the nuclear periphery with lesser amounts of label present inside the nuclei (Fig. 2H,K). As chromosomes began to decondense and the nuclear envelope reassembled the labeling of the Rev protein was limited to the cytoplasm and the nuclear interior was almost

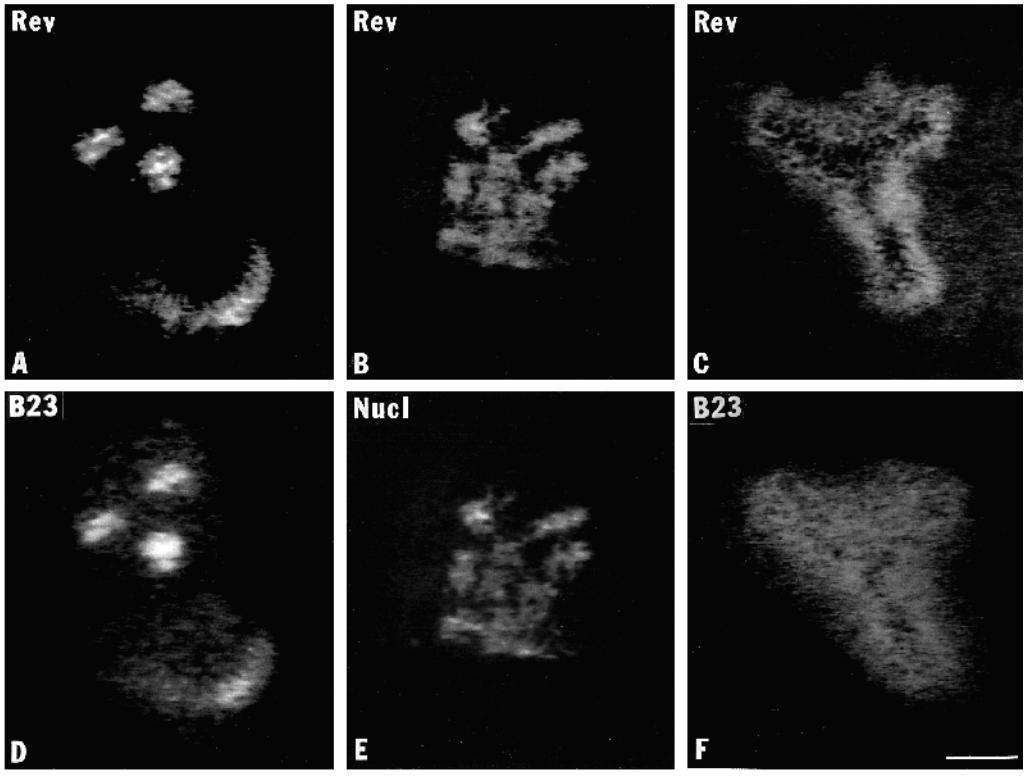

Fig. 3. Colocalization of the Rev protein with other nucleolar proteins during early stages of mitosis in Rev-expressing CMT3 cells. In subsequent figures (except Fig. 6) the upper panel of each pair of fluorescence micrographs shows localization of Rev and the lower panel shows localization of another antigen in the same optical section. In early prophase the Rev protein (A,B) colocalized with protein B23 (D) labeled with mAb anti-B23 antibody and nucleolin (E) visualized by polyclonal anti-nucleolin antibody in disintegrating nucleolar bodies. In prometaphase the Rev protein (C) is present in the perichromosomal region of each chromosome similar to the pattern seen with protein B23 (F). Bar, 10 μm.

negative for the Rev protein (see Figures below). In early G_1 phase when pre-rRNA transcription had fully restarted the Rev protein was predominantly located in the reformed nucleoli with negative staining of the nucleoplasm and occasional labeling in the cytoplasm (Fig. 2I,L). There were no significant differences in Rev protein behavior at each stage of mitosis if the Rev-expressing CMT3 cell line coexpressed the RRE-containing *gag-pol* RNA (not shown). The HIV-1 major core protein p24 whose expression was used as the marker of the Rev protein activity in these cells was localized exclusively in the cell periphery during each stage of mitosis (not shown). In summary, after nucleolar dispersal, the Rev protein appeared to be temporarily stored on the peripheral regions of chromosomes, then move to the MNF and eventually migrate to fully functional nucleoli.

Immunolocalization of the Rev protein during mitosis relative to several other nucleolar proteins

To determine the possible association of Rev with nucleolar proteins or subcellular components during mitosis, we performed colocalization studies using antibodies to Rev and to several nucleolar proteins in the Rev-expressing CMT3 cells. The behavior of the Rev protein in early and late prophase was virtually identical to that of protein B23, fibrillarin, nucleolin and the p52 protein of the granular components of nucleoli (GC). The release of the Rev protein from the disintegrating nucleolar bodies during late prophase was coincidental with the loss of protein B23 (Fig. 3A,D), nucleolin (Fig. 3B,E), fibrillarin and the p52 protein of the GC (not shown). Similarly, during prometaphase the Rev protein colocalized with nucleolar proteins such as protein B23 in the periphery of all chromosomes (Fig. 3C,F). In contrast, ribosomal protein S6 in Rev-expressing CMT3 cells was freely dispersed among the chromosomes in the cytoplasm (not shown). A pattern of localization similar to that of the Rev protein was seen for other nucleolar proteins in the metaphase plate (not shown) and in chromosomal peripheral labeling during anaphase for protein B23 (Fig. 4A,D), nucleolin (Fig. 4B,E), fibrillarin and the p52 protein of the GC (not shown). In early anaphase the Rev protein was also detectable in the numerous NDF in which protein B23 (Fig. 4D, arrows), nucleolin (Fig. 4E, arrows), fibrillarin and the p52 protein of the GC (not shown) were also located. To eliminate the possibility that these foci were artifacts of fixation other methods of fixation and permeabilization were used: the foci were always present under every fixation condition used. The NDF could be seen as dense structures by phase contrast microscopy (Fig. 4C,F, arrows). These NDF were observed in similar numbers in untransfected COS-7 cells and in lesser amounts in HeLa cells (not shown). Thus, they do not appear to be the result of transfection by the Rev-expressing plasmid. The NDF did not colocalize with the clusters of mitotic interchromatin granules (MIGs) detected by an antibody against splicing factor SC35 (Fig. 5). However, the NDF were frequently in close proximity with MIGs (Fig. 5C).

The colocalization of the Rev protein with nucleolar proteins in the chromosomal periphery continued into early telophase. During re-assembly of the nuclear envelope the Rev protein colocalized with the p52 protein of the GC (Fig. 6A,B), nucleolin (Fig. 6C,D), protein B23 and fibrillarin (not shown) among decondensing chromosomes in the proximity of the nuclear envelope. The nuclear interiors were nearly negative for Rev protein staining (Fig. 6A,C). The nucleolar organizer regions

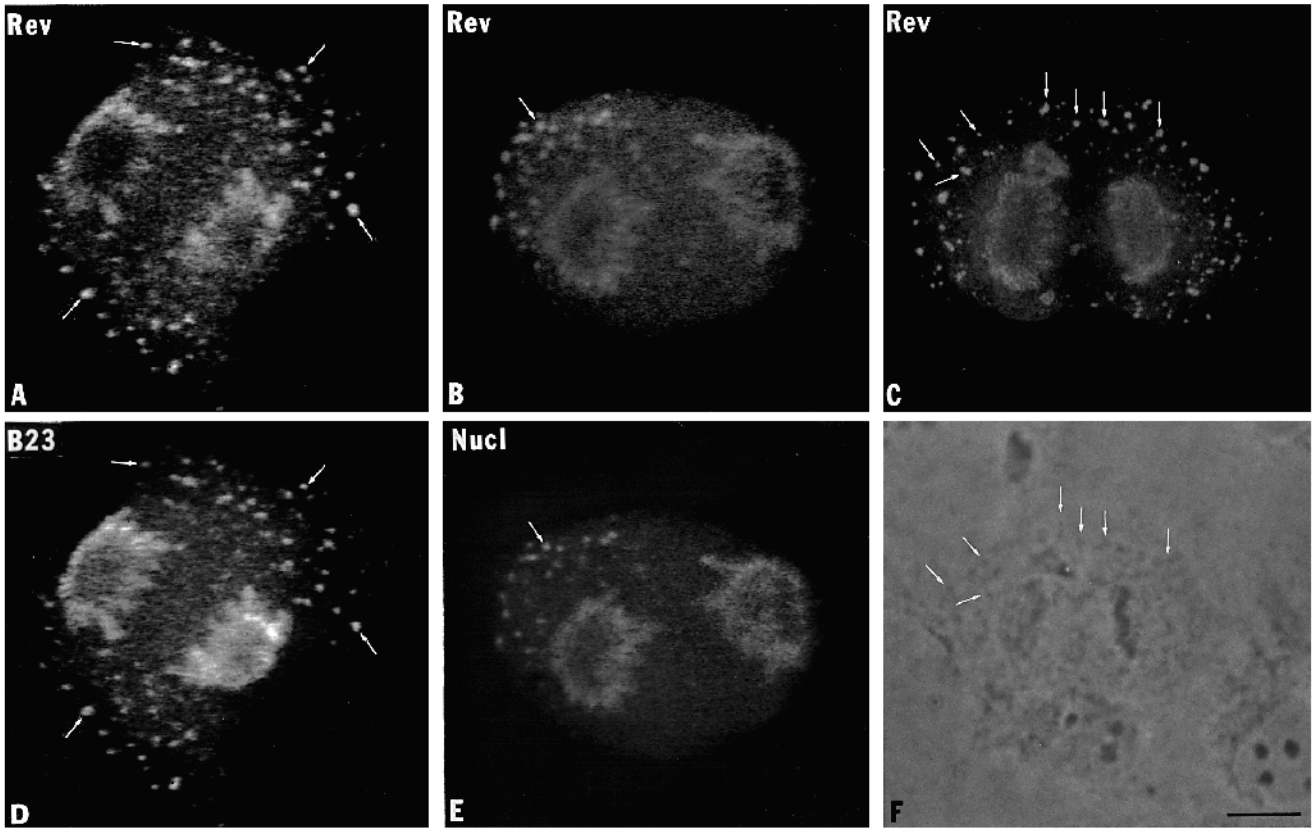

Fig. 4. Localization of the Rev protein and other nucleolar proteins during anaphase. The Rev protein decorates the perichromosomal region (A,B,C) as does protein B23 (D) stained by polyclonal anti-B23 antibody and nucleolin (E) stained by polyclonal anti-nucleolin antibody. The Rev protein is present in numerous nucleolus-derived foci (arrows) and colocalizes in these with protein B23 (D) and nucleolin (E). The nucleolus-derived foci can be seen as dense structures by phase contrast (F). Bar, 10 μm.

(NORs) which were stained by an antibody against the RNA polymerase (pol) I exhibited a pattern of compact fluorescent spots, some of which were located in the proximity of reforming nuclear envelope (Fig. 7A,D). Rev did not associate with these NOR markers. At this stage lamins were detected mostly in the cytoplasm by the anti-lamin antibody although some signal was found associated with the nuclear envelope (Fig. 7E). At the same time Rev was predominantly seen as a ring inside but overlap-

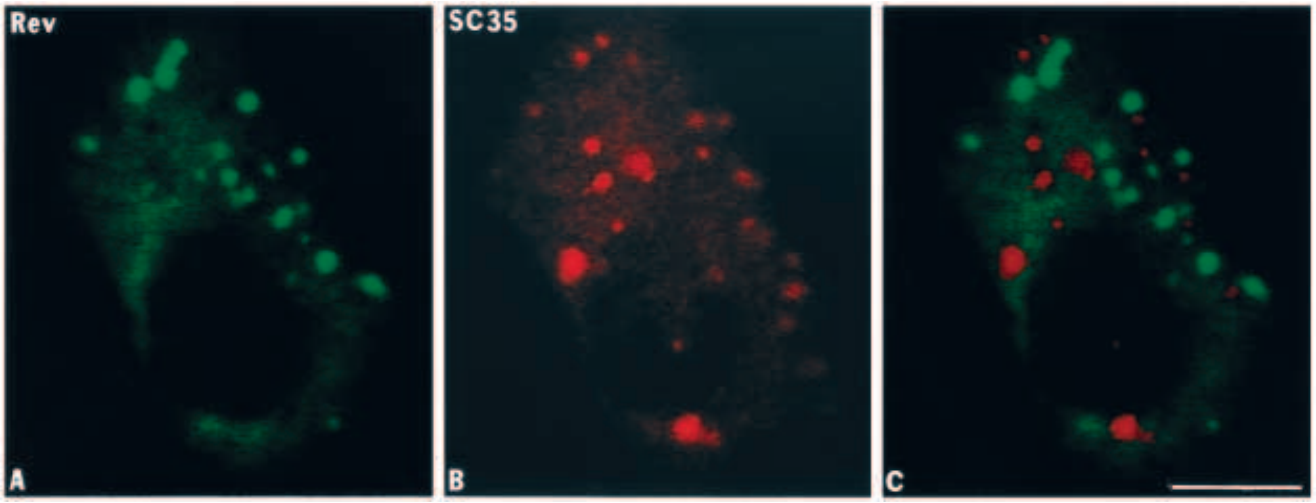

Fig. 5. Different subcellular distribution of Rev-positive nucleolus-derived foci and mitotic interchromatin granules (MIGs) in telophase cells. The localization pattern of Rev-positive foci (A) is not identical with the localization of clusters of MIGs visualized by mAb antibody against the splicing factor SC35 in late telophase cell (B). In C the images in A and B were superimposed to show that the two types of structures did not colocalize. Some foci are in close proximity with MIGs. Bar, 10 μm.

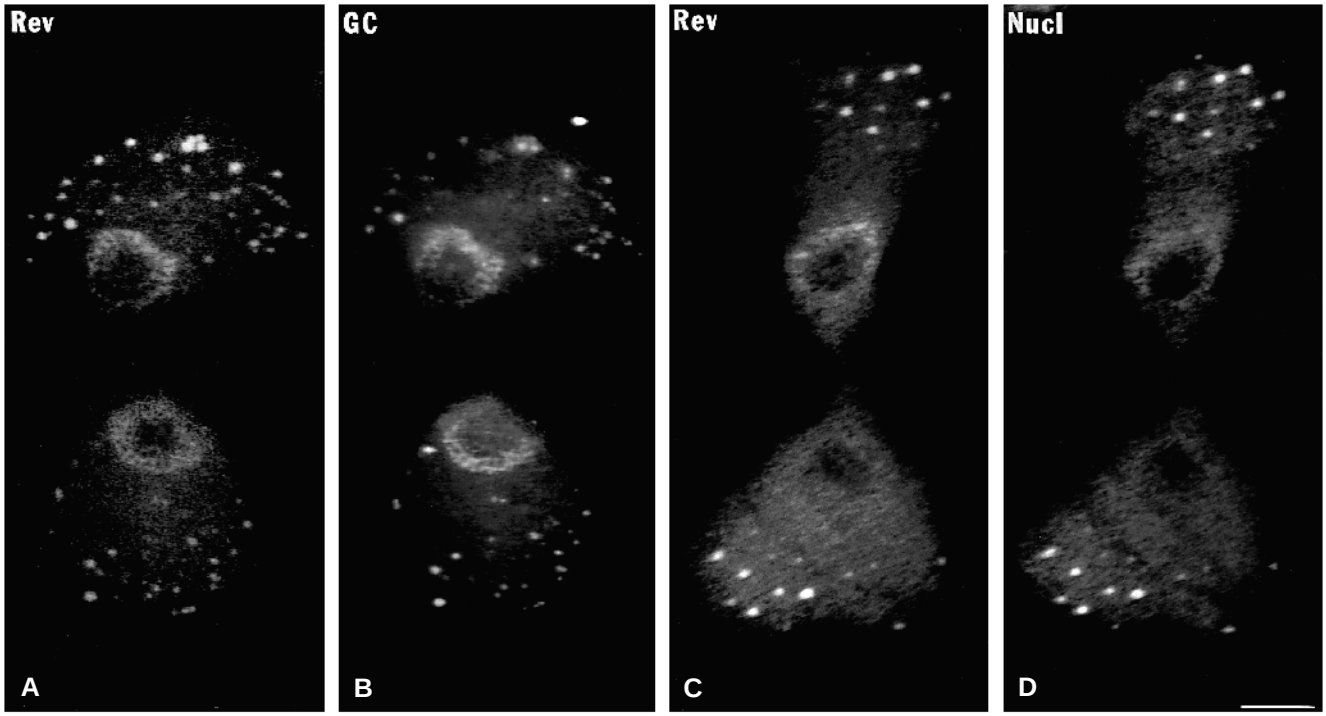

Fig. 6. Location of the Rev protein and other nucleolar proteins in early telophase. In this figure the left panel of each pair of micrographs shows the location of Rev and the right panel shows the location of another antigen. The Rev protein is in close proximity to decondensing chromosomes and it is still abundant in prominent nucleolus-derived foci (A,C) where it colocalizes with protein p52 of the GC (B) labeled with G04 autoantibody and nucleolin (D) stained by polyclonal anti-nucleolin antibody. Bar, 10 μm.

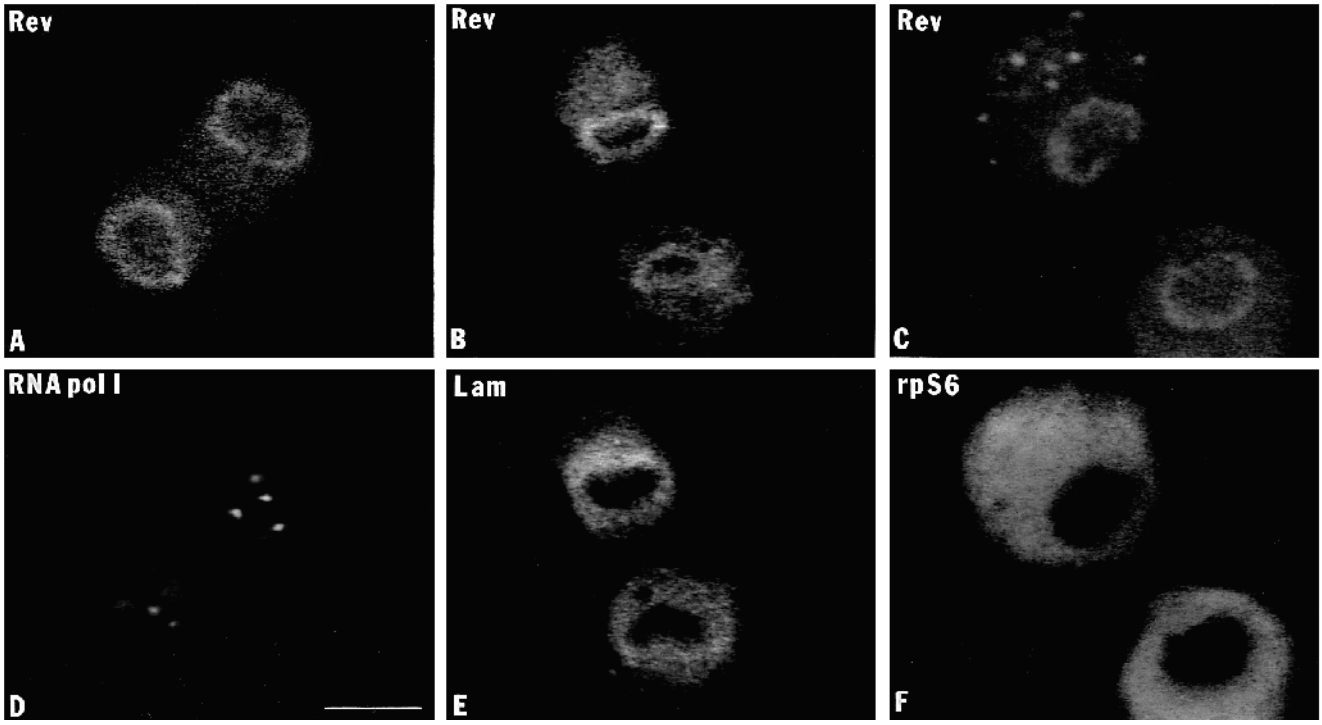

Fig. 7. Location of the Rev protein and other proteins in early telophase cells. The Rev protein in close association with decondensing chromosomes when the nuclear envelope is formed in early telophase cells (A) does not colocalize with the NORs visualized with antibody against RNA pol I (D). The distribution of the Rev protein associated with the decondensing chromosomes in early telophase (B) is different from the diffuse cytoplasmic localization of lamins stained by a polyclonal anti-lamin antibody (E). Similarly, ribosomal protein S6 is located in the cytoplasm in telophase (F) and excluded from the decondensing chromosome area in the nuclear periphery stained with anti-Rev mAb antibody (C). Bar, 10 μm.

ping with the region stained by the anti-lamin antibody (Fig. 7B). Ribosomal protein S6 staining was excluded from the nuclear interior and remained dispersed in the cytoplasm (Fig. 7C,F).

After completion of nuclear envelope reassembly and nucleocytoplasmic compartmentalization protein B23 (Fig. 8D), fibrillarin, nucleolin and the p52 protein of the GC (not shown) were present in numerous small prenucleolar bodies (PNBs) (Fig. 8D, small arrows) and in the reformed nucleoli (Fig. 8D,E, large arrows) of daughter cells. In contrast, the Rev protein was excluded from the nuclei to the cytoplasm (Fig. 8A,B). The positions of PNBs were clearly distinct from the NORs detected by the antibody against RNA pol I (not shown). At this stage the number of NDF dramatically decreased to a few or none (Fig. 8A,B). The redistribution of the NDF to the close proximity of reassembling nuclear envelopes of daughter cells during telophase as shown with nucleolar remnants by Azum-Gélade et al. (1994) was seen in CMT3 cells only occasionally (Fig. 8B,E, arrowheads).

The *trans*-dominant negative Rev protein follows a different import pathway into the daughter nuclei from the wild-type Rev protein

Immunofluorescence localization studies were also performed on CMT3 cells expressing Rev in which two amino acids were deleted from the NES (pCMVrevD78-79). The Rev protein expressed from pCMVrevD78-79 has a *trans*-dominant negative effect on Rev function. This has been demonstrated in transient experiments and after transfection of the 2AB7 cell line with Rev-dependent HIV-1 constructs. In these experiments it was shown that HIV-1 gag/pol expression from constructs containing the HIV-1 RRE was efficiently inhibited by *trans*-dominant negative Rev D78-79 (TD Rev). The inhibition was as efficient as that previously observed with the Rev M10 protein (Malim et al., 1989). The results of these experiments will be described elsewhere (M.-L. Hammarskjöld et al., unpublished).

Initial studies were done to determine whether the ability of TD Rev to migrate from the nucleus to the cytoplasm is impaired as it is in other *trans*-dominant mutants of Rev (M10, M27, M28, M29, M32) after blocking transcription with actinomycin D (AMD) (Meyer and Malim, 1994; Szilvay et al., 1995; Wolff et al., 1995). The TD Rev-expressing CMT3 cells were treated with 0.5 μg/ml of AMD in time course experiments. This concentration of AMD induced complete redistribution of the wild-type Rev protein in CMT3 cells from the nucleus to the cytoplasm in 50 minutes (Dundr et al., 1995),

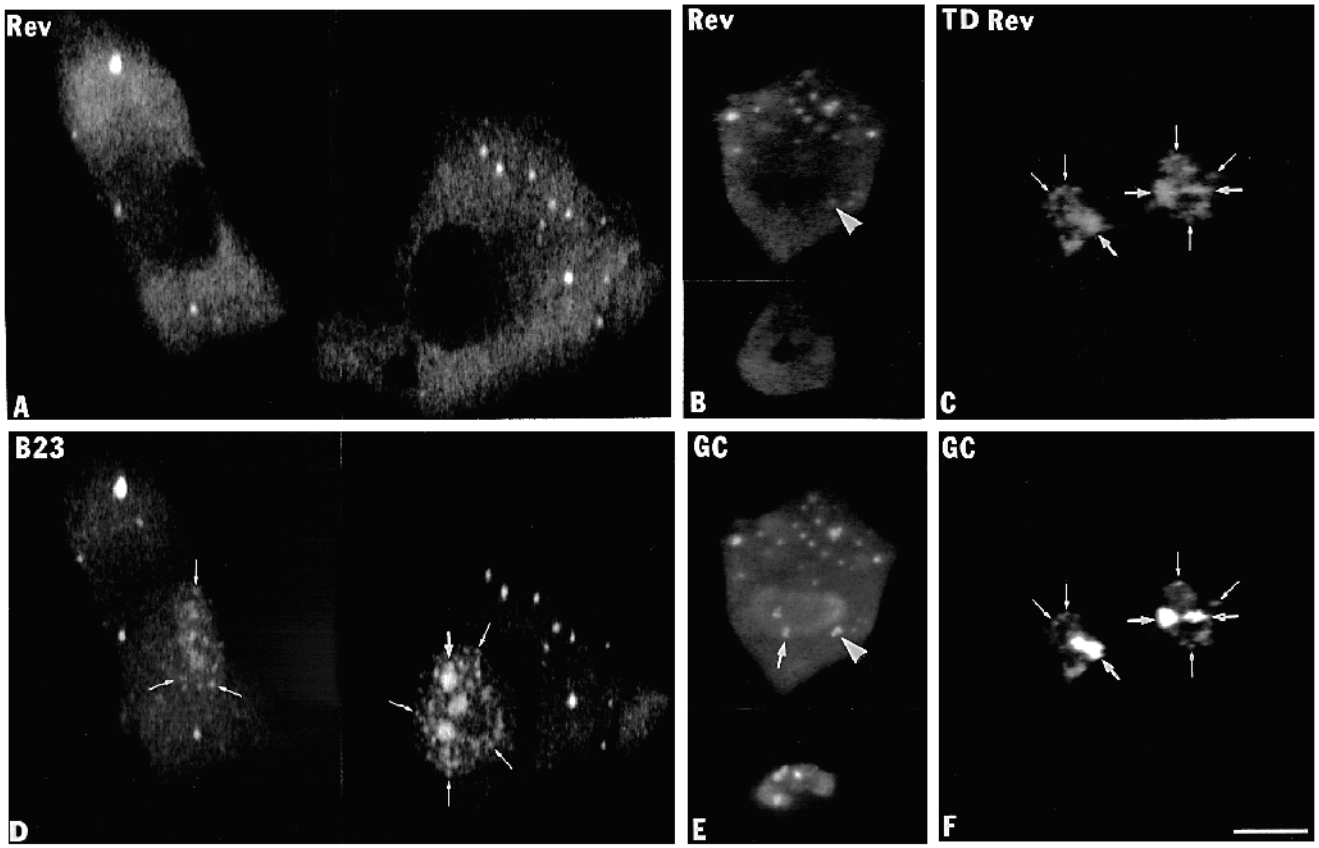

Fig. 8. Localization of the Rev protein and nonribosomal nucleolar proteins in late telophase cells. In late telophase when the nuclear envelope is formed the wild-type Rev protein (A,B) is not seen in the nuclear interior where protein B23 is present in numerous prenucleolar bodies (D, small arrows) and in reformed nucleoli (D, large arrows) labeled with monoclonal anti-B23 antibody. Both proteins are clearly visible in several nucleolus-derived foci (A,D). These were occasionally seen adjacent to the nuclear envelope (B,E, arrowheads) Similarly, the wild-type Rev protein is not detectable inside of nuclei in late telophase (B) where the p52 protein of the GC is located in reformed nucleoli (E, arrow) and in the nucleoplasm around the reformed nuclear envelope (E). Both proteins colocalized in nucleolus-derived foci (B, E). In contrast, the *trans*-dominant negative mutant Rev protein is located in PNBs (C, small arrows) and also in nucleoli (C, large arrows) as is the p52 protein of the GC (F) in late telophase. Bar, 10 μm.

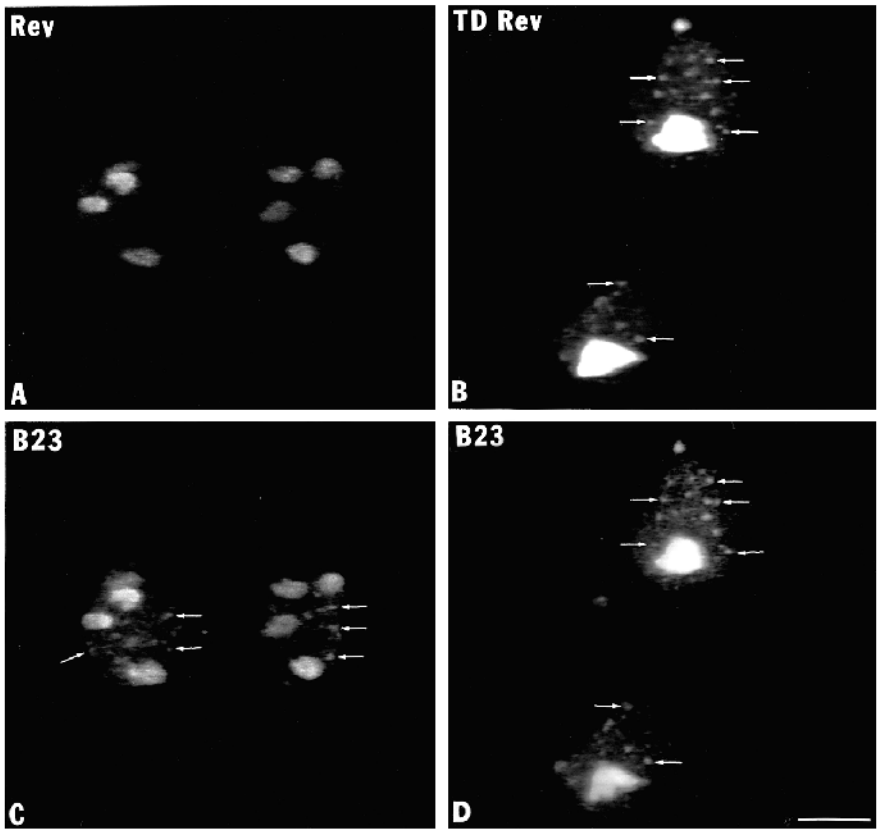

Fig. 9. Subcellular localization of wild-type Rev in early G_1 phase and *trans*-dominant negative mutant Rev in late telophase and early G_1 phase. In early G_1 phase, the wild-type Rev protein is localized in the reformed postmitotic nucleoli (A) whereas protein B23 is present in PNBs (C, arrows) and in nucleoli. In early G_1 phase the *trans*-dominant negative Rev protein is predominantly present in fully reformed nucleoli (B) but it is also located in PNBs (B, arrows) where it colocalizes with protein B23 (D). Bar, 10 μm.

but the TD Rev protein remained partly in segregated nucleoli and shifted to the nucleoplasm within 3 hours but did not move to the cytoplasm (not shown). Thus, this TD Rev protein has characteristics similar to other nonfunctional mutants of the Rev protein in that it does not shuttle from the nucleus to the cytoplasm (Meyer and Malim, 1994; Szilvay et al., 1995; Wolff et al., 1995).

The TD Rev protein showed a pattern of localization identical to that of the wild-type Rev protein at the chromosome periphery after nucleolar disintegration and in the NDF during mitosis. However, in late telophase when the nuclear envelope has reformed and the chromosomes have decondensed the TD Rev appeared in PNBs (Fig. 8C, small arrows) and in the reformed nucleoli (Fig. 8C, large arrows) in a manner similar to that of nonribosomal nucleolar proteins, e.g. the p52 protein of the GC (Fig. 8F). In contrast, the wild-type Rev protein reentered the postmitotic nuclei later after their formation. When we treated the cells synchronized in mitosis with AMD at a dose that inhibits the transcription of RNA pol I and II (1 μg/ml) and at a dose that inhibits only RNA pol I (0.04 μg/ml) for 45 minutes (Piñol-Roma and Dreyfuss, 1991), we found that inhibition of RNA pol I and II as well as only RNA pol I prevents the nuclear import of the Rev protein into postmitotic nuclei (not shown). These results indicate that the postmitotic nuclear targeting of the wild-type Rev is also dependent on RNA pol I transcription as was found with interphase cells.

The wild-type Rev protein appeared in nearly completely formed nucleoli along with protein B23 (Fig. 9A,C) and other nucleolar proteins (not shown) in early G_1 phase. In this case, numerous PNBs were labeled for protein B23 (Fig. 9C, arrows), nucleolin, fibrillarin and the p52 protein of the GC in the nucleoplasm of daughter cells but not for Rev (Fig. 9A). However, the nuclei of early G_1 phase cells expressing the TD Rev protein also contained PNBs positive for the TD Rev and protein B23 (Fig. 9B,D, arrows) and other nucleolar proteins (not shown). Interestingly, when the wild-type Rev protein was clearly detectable in compact daughter nucleoli (Fig. 10A, arrows), ribosomal protein S6 was exclusively located only in the cytoplasm (Fig. 10B) suggesting that Rev accumulates in the nucleoli before at least some ribosomal proteins arrive. At this point in the cell cycle, the wild-type Rev protein was predominantly located in the nucleoli and was only occasionally seen in the cytoplasm. Thus, the wild-type Rev protein's appearance in the postmitotic nucleoli was not associated with the first and second steps of nucleolar reformation; i.e. incorporation into PNBs followed by their fusion with transcriptionally active NORs. In contrast, the TD Rev protein reentered the nuclei by the pathway normally followed by nonribosomal nucleolar proteins; i.e. association with PNBs which then aggregate around the NORs of the developing nucleoli.

DISCUSSION

In this study, we have used stable Rev-expressing CMT3 cell lines to show that the wild-type Rev protein and a *trans*-dominant negative Rev (TD Rev) exhibit behavior typical of nonribosomal nucleolar proteins until telophase. These proteins leave disintegrating nucleoli during late prophase,

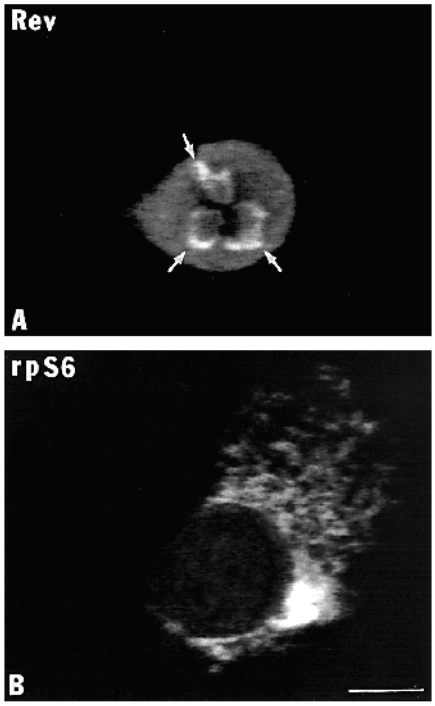

Fig. 10. Subcellular localization of the wild-type Rev and ribosomal protein S6 in early G_1 phase. The Rev protein (A) is predominantly localized in the reformed postmitotic nucleoli (A, arrows) but ribosomal protein S6 (B) is only present in the cytoplasm at this time. Bar, 10 μm.

move to the surfaces of chromosomes during prometaphase and remain largely associated with chromosomes until early telophase. In addition, they accumulate in numerous nucleolus-derived foci (NDF) during anaphase and telophase. However, when the nuclear envelope reassembles and compartmentalization into nucleus and cytoplasm is reestablished, different pathways are taken by the wild-type Rev and nonribosomal nucleolar proteins. At this stage the wild-type Rev protein is excluded from the nucleus whereas other nonribosomal nucleolar proteins and the TD Rev appear in prenucleolar bodies (PNBs) which coalesce around the chromosomal NORs of the developing nucleolar body. Thus, the Rev protein's reentry into the reforming nucleolus is delayed until early G_1 phase and lags behind other nonribosomal nucleolar proteins. The two pathways are illustrated diagrammatically in Fig. 11. The timing of Rev's reappearance in the nucleolus appears to depend on reestablishment of rRNA transcription and its continuation. Interestingly, ribosomal protein S6 import is even further delayed even though it has a nucleolar targeting signal remarkably similar to that of Rev (Schmidt et al., 1995).

There are five clearly defined fates for the components of the nucleus following the mitosis-induced disassembly (Earnshaw and Bernat, 1991; Rattner, 1992; Chaudhary and Courvalin, 1993; Hernandez-Verdun and Gautier, 1994; Bregman et al., 1995; Spector et al., 1995): (1) some nuclear proteins (e.g. chromatin-associated proteins) associate with the spindle microtubules during prophase and prometaphase and segregate into the daughter cells at the metaphase/anaphase transition. (2) Proteins of the nuclear envelope, including lamins, diffuse into the cytoplasm and are distributed passively into the daughter cells. (3) Some proteins are incorporated into mitotic interchromatin granules including splicing proteins of the Sm snRNP and SerArg families and RNA pol II. (4) 'Chromosomal passenger proteins' are transiently associated with mitotic chromosomes during prometaphase and metaphase and then become closely associated with the microtubules of the central spindle at the onset of anaphase. (5) Proteins that disperse to the perichromosomal layer during prometaphase until early telophase are generally nuclear matrix- and nucleolar proteins. Our results demonstrate that the Rev protein belongs to the fifth group of proteins because it leaves the perichromosomal layer when the nuclear envelope starts to reform.

The component(s) in the chromosome periphery to which Rev is bound have not been identified but they could be protein, DNA or RNA. The perichromosomal regions have been shown to be completely negative for ribosomal RNA (rRNA) (Jiménez-García et al., 1994; Weisenberger and Scheer, 1995). Thus, it is unlikely that Rev is bound to the chromosome periphery through interactions with rRNA. On the other hand these regions contain small nucleolar RNP particles which could form complexes with Rev and other proteins (Moyne and Garrido, 1976; Medina et al., 1995).

The translocation and targeting mechanisms of these nucleolar components from the disintegrating nucleoli to the perichromosomal region and the regulation of their movement during prophase are still unknown, but a phosphorylation-dephosphorylation cycle may be involved (Peter et al., 1990). However, there is no evidence that the Rev protein is specifically phosphorylated or dephosphorylated during mitosis and, to date, the Rev protein has been shown to be phosphorylated in vivo only by protein kinase C (Hauber et al., 1988). Our unpublished studies indicate the Rev protein is not degraded and resynthesized during mitosis unlike certain other nucleolar proteins; e.g. p130 (Pai et al., 1995). The perichromosomal layer may be a transient storage site that permits efficient redistribution of preexisting nuclear and nucleolar proteins equally between daughter cells, which also prevents mixing of particular nuclear constituents with the cytoplasm before nuclear envelope reformation and thus ensures immediate incorporation into the newly formed nuclei (Benavente et al., 1989b; Hernandez-Verdun et al., 1994).

During early anaphase when the chromosomes are pulled toward the spindle poles, the wild-type Rev protein and the TD Rev appear in numerous NDF located outside the mitotic spindle region. The NDF may be the same as the nucleolar remnants (NRs) originally observed by Hsu et al. (1965) and later by Noel et al. (1971). More recently, the NRs were shown to contain nucleolar perichromosomal proteins and U3 snoRNA (Azum-Gélade et al., 1994; Gautier et al., 1994). They are also characteristic of highly proliferating cells with large nucleoli such as CHO cells (Azum-Gélade et al., 1994; Gautier et al., 1994). Interestingly, the CHO cells of the above studies contained limited numbers of NRs compared to the NDF of CMT3 cells. How the NDF arise is not clear; however, their large size (up to 2 μm) suggests they result from the fusion of substantial amounts of nucleolar material that is released from the chromosome surfaces. The decreasing number of NDF as the cells progress to late stages of mitosis also suggests that they serve as a source of proteins that become incorporated into nucleoli (Azum-Gélade et al., 1994).

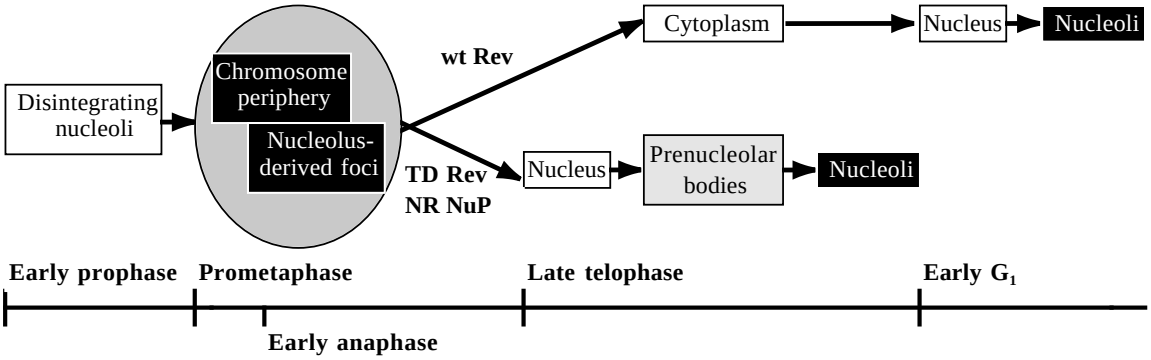

Fig. 11. Schematic diagram of the behavior of wild-type Rev and TD mutant Rev proteins during mitosis. The wild-type Rev and TD Rev proteins are released from the disintegrating nucleoli during prophase and are redistributed to the chromosome periphery during prometaphase. The wild-type Rev and TD Rev are also present in numerous nucleolus-derived foci which are detectable in early anaphase. When the nuclear envelope is reformed the wild-type Rev protein is excluded from the decondensed chromosome periphery to the cytoplasm and it is imported to the postmitotic nucleus and the nucleoli in early G_1 phase. In contrast, the TD Rev protein follows the pathway of nonribosomal nucleolar proteins (NR NuP) after nuclear envelope reformation in nucleologenesis. The TD Rev protein is present in prenucleolar bodies which fuse with transcriptionally active NORs to become nucleoli in late telophase.

In any event, these particles seem to be a major site of storage for the Rev protein before it reenters the nucleus.

In early telophase, the beginning of nuclear envelope and nucleolar reformation approximately coincides with the appearance of numerous PNBs that contain nonribosomal proteins and U3 snRNP. These components are also found in the DFCs of interphase nucleoli (Bell et al., 1992; Scheer et al., 1993; Jiménez-García et al., 1994). The PNBs rapidly coalesce around the chromosomal NORs that have begun transcribing pre-rRNA which is incorporated into developing DFCs. The PNBs are believed to provide a mechanism for rapid assembly of preexisting nucleolar proteins into the nucleolus before it is fully operational in ribosome biogenesis.

A striking finding of this study was that the wild-type Rev protein does not follow the normal route to the nucleolus, whereas the TD Rev protein does; i.e. import of the wild-type Rev protein into the nucleus and nucleoli is delayed until early G_1 phase and bypasses the PNBs when it does enter nucleoli. How can the different behaviors of the wild-type and TD Rev be explained? The TD Rev protein is unable to induce nuclear export of incompletely spliced viral transcripts containing the RRE from the nucleus to the cytoplasm and is unable to shuttle between the nucleus and the cytoplasm like other *trans*-dominant negative Rev mutants (Malim et al., 1989; Meyer and Malim, 1994; Kalland et al., 1994; Wolff et al., 1995). Although these mutants can bind to the RRE, critical alterations in the leucine-rich activation domain of Rev block its interaction with the nuclear export system. This leucine-rich domain was recently shown to be the nuclear export signal (Malim and Cullen, 1991; Bogerd et al., 1995; Fischer et al., 1995; Fritz et al., 1995; Stutz et al., 1995; Wen et al., 1995; reviewed by Gerace, 1995; Görlich and Mattaj, 1996). Functionally equivalent NESs have recently been identified in TFIIIA (Fischer et al., 1995) and PKI, an inhibitor of cAMP-dependent protein kinase (Wen et al., 1995). By the yeast two-hybrid system two groups independently isolated the same ~58 kDa human protein interacting with the Rev NES called Rab (Bogerd et al., 1995) or hRIP (Fritz et al., 1995). Immunofluorescence microscopy showed Rab to be located in nucleoli as well as in the nucleoplasm (Bogerd et al., 1995), whereas hRIP was found in the nucleoplasm and at the nuclear pore complexes (Fritz et al., 1995). Stutz et al. (1995) also identified a ~42 kDa yeast protein Rip1p that interacts with the Rev NES and is localized at the nuclear pore complexes. The sequences of these two proteins have common multiple FG repeats which are also present in the nucleoporins (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). In competition experiments it was discovered that Rev-mediated export of viral RNAs competes with the nuclear export of 5S rRNA and U snRNAs, which bind proteins that are functionally equivalent to the Rev protein and interact with the same or analogous nuclear export factor (Fischer et al., 1995).

It is possible that the wild-type Rev protein interacts through its NES with a nuclear export factor almost immediately after its release from the perichromosomal layer. It is known that the perichromosomal proteins leave the chromosomes at the same time that the integral proteins of the inner nuclear membrane are targeted to chromosomes in early telophase (Hernandez-Verdun and Gautier, 1994). Thus a Rev-nuclear export factor complex may interact with nuclear pore components and be excluded to the cytoplasm as soon as the nuclear pore complexes have been reconstituted. However, the current studies do not permit us to distinguish between simple exclusion and active nuclear export.

Our previous and current work indicates that nuclear targeting of Rev to interphase nuclei depends on continuing RNA pol I transcription (Dundr et al., 1995). Consistent with this, the wild-type Rev protein cannot be retained in postmitotic nuclei until preribosomal RNA transcription reestablished at a high level in early G_1 phase. This may explain why NES-mediated nuclear export in telophase dominates over NLS-mediated nuclear import beginning immediately after nuclear envelope reformation (Benavente et al., 1989a,b). Thus the wild-type Rev protein appears to enter the nucleus only when fully active nucleoli are present. In contrast, the TD Rev protein is unable to interact with a nuclear export factor, but it is able to bind nucleolar proteins (e.g. protein B23) through its basic NOS domain to be targeted to PNBs and subsequently to partially reformed nucleoli. An alternative explanation is that in the wild-type Rev the NOS-protein interaction is blocked in some way by another factor

interacting with the NES. This could cause the wild-type Rev protein to remain in the cytoplasm until the factor is removed. In this case also, the TD Rev protein would passively follow nucleolar proteins through the nucleolar reformation process. In any event the nuclear import pathway seems to be modulated by Rev's interaction with the nuclear export factor. To understand the precise mechanism by which this takes place will require a more thorough comprehension of nucleocytoplasmic transport as well as nucleologenesis.

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