# Differential Interaction of Splicing snRNPs with Coiled Bodies and Interchromatin Granules during Mitosis and Assembly of Daughter Cell Nuclei

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*Abstract.* In the interphase nucleus of mammalian cells the U1, U2, U4/U6, and U5 small nuclear ribonucleoproteins (snRNPs), which are subunits of spliceosomes, associate with specific subnuclear domains including interchromatin granules and coiled bodies. Here, we analyze the association of splicing snRNPs with these structures during mitosis and reassembly of daughter nuclei. At the onset of mitosis snRNPs are predominantly diffuse in the cytoplasm, although a subset remain associated with remnants of coiled bodies and clusters of mitotic interchromatin granules, respectively. The number and size of mitotic coiled bodies remain approximately unchanged from metaphase to early telophase while snRNP-containing clusters of mitotic interchromatin granules increase in size and number as cells progress from anaphase to telophase. During telophase snRNPs are transported into daughter nuclei while the clusters of mitotic interchromatin granules remain in the cytoplasm. The timing of nuclear import of splicing snRNPs closely correlates with the onset of transcriptional activity in daughter nuclei. When transcription restarts in telophase cells snRNPs have a diffuse nucleoplasmic distribution. As cells progress to G1 snRNP-containing clusters of interchromatin granules reappear in the nucleus. Coiled bodies appear later in G1, although the coiled body antigen, p80 coilin, enters early into telophase nuclei. After inhibition of transcription we still observe nuclear import of snRNPs and the subsequent appearance of snRNP-containing clusters of interchromatin granules, but not coiled body formation. These data demonstrate that snRNP associations with coiled bodies and interchromatin granules are differentially regulated during the cell division cycle and suggest that these structures play distinct roles connected with snRNP structure, transport, and/or function.

SPLICING of pre-mRNAs in the nucleus of mammalian cells requires a complex set of factors which interact in an ordered pathway to form the spliceosome (for recent reviews see Green, 1991; Lamond, 1993; Moore et al., 1993). Major components of the spliceosome are the U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs).[1] In addition, a number of non-snRNP splicing factors have been recently identified (reviewed by Lamm and Lamond, 1993). Each spliceosomal snRNP contains a single snRNA species and a common set of core proteins, as well as unique specific proteins (reviewed by Lührmann et al., 1990; Will et al., 1993).

To understand the organization and assembly of the splicing apparatus in vivo, a number of studies have addressed the localization of spliceosomal snRNPs in the nucleus of mammalian cells. For example, indirect immunofluorescence has been performed using antibodies directed against either snRNP proteins, or antibodies against the snRNA-specific 2,2,7-trimethylguanosine (m3G) cap structure. This has revealed that snRNPs are diffusely distributed in the nucleoplasm and in addition concentrate in 20–50 subnuclear structures, producing an overall "speckled" pattern (Habets et al., 1989; Nyman et al., 1986; Reuter et al., 1984; Spector, 1984; Verheijen et al., 1986). More recently, antisense probes have been used to specifically target each spliceosomal snRNA by in situ hybridization (Carmo-Fonseca et al., 1991*a*,*b*, 1992; Huang and Spector, 1992; Matera and Ward, 1993; Visa et al., 1993*a*). The combined approach of double labeling with antisense probes to snRNAs and antibodies against snRNP-specific proteins has confirmed that fully assembled snRNPs are being detected in these studies. Fluorescence microscopy data from several laboratories indicate that the U1snRNP tends to be more widespread in the nucleoplasm, whereas the U2, U4/U6, and U5 snRNPs have a more pronounced "speckled" distribution pattern (Carmo-

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1. *Abbreviations used in this paper*: CB, coiled bodies; IG, interchromatin granules; MCB, mitotic coiled bodies; MIG, mitotic interchromatin granules; snRNPs, small nuclear ribonucleoprotein particles.

Fonseca et al., 1991*a*,*b*, 1992; Matera and Ward, 1993). This is supported by EM analysis showing that specific subnuclear regions contain U1 but not the other splicing snRNPs (Visa et al., 1993*a*).

The punctate or "speckled" snRNP distribution observed by fluorescence microscopy results from the association of snRNPs with several distinct subnuclear structures, including perichromatin fibrils, interchromatin granules (IGs) and coiled bodies (CBs) (Fakan et al., 1984, 1986; Puvion et al., 1984; Visa et al., 1993*a*; for recent reviews see Brasch and Ochs, 1992; Lamond and Carmo-Fonseca, 1993*a*,*b*). It is likely that the association of snRNPs with perichromatin fibrils account, at least in part, for the diffuse nucleoplasmic staining observed in the fluorescence microscope. However, this has still to be conclusively established, since no antibody markers specific for perichromatin fibrils have as yet been characterized. In contrast, both IGs and CBs can be precisely identified by fluorescence microscopy using specific antibodies (Turner and Franchi, 1987; Raska et al., 1990, 1991; Andrade et al., 1991). This has allowed a clear demonstration that within the speckled staining pattern, splicing snRNPs colocalize in both IGs and CBs.

Several studies have demonstrated that the pattern of snRNP distribution within the nucleus varies between different cell types (Spector et al., 1992; Matera and Ward, 1993), and, in the same cell line, can be altered by factors that affect mRNA synthesis (Carmo-Fonseca et al., 1992, 1993; Antoniou et al., 1993) and viral infection (Martin et al., 1987; Phelan et al., 1993; Jiménez-Garcia and Spector, 1993; Bridge et al., 1993). An important corollary of these experiments is that the organization of splicing snRNPs within the nucleus is dynamic. The level of snRNP associating with the separate subnuclear structures may reflect differences in the intracellular rates of snRNP assembly/disassembly and RNA metabolism or transport (for further discussion see Lamond and Carmo-Fonseca, 1993*a*,*b*).

How are snRNPs organized during mitosis, when transcription and processing of RNA cease and the nucleus breaks down? Previous immunofluorescence studies, using antibodies directed against either common snRNP proteins (i.e., "Sm" antigens), or the U1 snRNP-specific 70K protein, revealed that snRNPs become widely distributed in the cytoplasm at metaphase and anaphase and re-enter the newly formed nuclei of the two daughter cells at telophase (Reuter et al., 1985; Spector and Smith, 1986; Verheijen et al., 1986; Leser et al., 1989; Spector et al., 1991). Immunoprecipitation and double-labeling studies with anti-m3G cap antibodies also showed that snRNAs do not dissociate from snRNP proteins during mitosis (Reuter et al., 1985). Using a monoclonal antibody against the UlsnRNP-70K protein, Verheijen et al. (1986) reported a diffuse staining of the mitotic cytoplasm, with a speckled pattern observed in late anaphase and telophase. The UlsnRNP speckles in the cytoplasm were also shown to be double labeled by anti-Sm antibodies. Leser et al. (1989) have studied the ultrastructural distribution of snRNPs during mitosis and observed that snRNP antigens are contained in clusters which are morphologically similar to interchromatin granules. There is not general agreement on this point, however, since other workers have observed snRNP antigens diffusely distributed in mitotic cells without detecting any snRNP-containing clusters in the cytoplasm (Spector et al., 1991).

To help understand the function of the separate subnuclear snRNP compartments, we have analyzed here the interaction of splicing snRNPs with IGs and CBs during and after mitosis. In addition, we have focused on the process of daughter nuclei reformation during telophase in order to correlate the onset of transcriptional activity with the nuclear import of splicing snRNPs and the assembly of CBs and IGs. The data indicate that the formation of IGs and CBs, as well as their interaction with snRNPs, are differentially regulated throughout the cell division cycle.

## Materials and Methods

### Cell Culture

HeLa and Hep 2 cells were grown on glass coverslips in Minimum Essential Medium supplemented with 1% glutamine, 10% fetal calf serum, and antibiotics. In some experiments the coverslips were coated with 1 mg/ml poly-L-lysine (Sigma Chemical Co., St. Louis, MO). For drug experiments fresh medium was added and allowed to equilibrate for 1 h. HeLa cells were then exposed to either actinomycin D at a concentration of 5 μg/ml or 0.04 μg/ml or 5,6-dichlorobenzimidazole riboside at a concentration of 75 mM, for 45–50 min before fixation.

### Immunofluorescence and In Situ Hybridization

Cells grown on coverslips were rinsed twice with PBS and fixed/permeabilized according to one of the following protocols: (*a*) cells were fixed with 3.7% paraformaldehyde in CSK buffer (Fey et al., 1986) for 10 min, washed 3× in PBS and subsequently extracted with 0.2% SDS for 10 min at room temperature (Carmo-Fonseca et al., 1992); and (*b*) cells were fixed/extracted with 3.7% paraformaldehyde in 65 mM Pipes, 30 mM Hepes, pH 6.9, 10 mM EGTA, 2 mM $MgCl_2$, and 0.5% Triton X-100 for 15 min at room temperature. After fixation/extraction the coverslips were extensively washed in PBS and either immediately used or kept in PBS at 4°C for up to 3 d.

Immunofluorescence and in situ hybridization with biotinylated 2′-*o*-alkyl oligoribonucleotides were performed as previously described (Carmo-Fonseca et al., 1992). Splicing snRNPs were labeled with the anti-Sm monoclonal antibody Y12 (Pettersson et al., 1984), anti-Sm human autoimmune serum "Küng," anti-m3G cap monoclonal antibody (Bochnig et al., 1987), anti-70K protein monoclonal antibody (Billings et al., 1982), and anti-B″ protein monoclonal antibody 4G3 (Habets et al., 1989).

Biotinylated 2′-*o*-alkyl oligoribonucleotide probes were used to label the U1 and U2snRNAs, as previously described (Carmo-Fonseca et al., 1992). The splicing factor SC-35 was detected with anti-SC-35 monoclonal antibody (Fu and Maniatis, 1990). HnRNP C and A1 proteins were revealed by monoclonal antibodies 4F4 and 4B10, respectively (Choi and Dreyfuss, 1984). Coilin was detected using rabbit polyclonal antibodies raised against a β-galactosidase fusion protein containing the carboxy-terminal region of p80 coilin (Andrade et al., 1991). Fibrillarin was labeled using monospecific human autoantisera, affinity-purified rabbit antibodies raised against a carboxy-terminal peptide of human fibrillarin (Jansen et al., 1991) and the monoclonal antibody 72B9 (Reimer et al., 1987). Proteins localized to interchromatin granules were detected with the monoclonal antibody 3C5 (Turner et al., 1987). Note that double-labeling with the 3C5 and the other monoclonal antibodies used was possible because 3C5 is IgM and all others are IgG. Specificity of double-labeling using monoclonal 3C5 (IgM) and other mouse IgGs was controlled by reacting cells with either primary antibody alone, followed by incubation with either anti-IgM or anti-IgG fluorochrome conjugates.

### Visualization of Transcription Sites

Visualization of transcription sites was performed according to Jackson et al. (1993) in HeLa cells grown on coverslips and permeabilized with 0.05% Triton X-100. Alternatively, cells were treated according to Wansink et al. (1993). In control experiments α-amanitin (50 μg/ml) or actinomycin D (5–10 μg/ml) were present during the transcription reaction.

### Fluorescence Microscopy

Samples were examined in a fluorescence microscope (Zeiss IM35 and

Zeiss Axiophot) using a 100X objective. Images were recorded with a Hamamatsu SIT-camera and either a DVS-3000 or ARGUS 10 image processor (Hamamatsu Photonics). Confocal microscopy was performed using both the EMBL compact confocal microscope (Stelzer et al., 1991) and the Zeiss LSM equipped with an argon ion laser (488 nm) and a HeNe laser (543 nm).

### Electron Microscopy

Immuno-electron microscopy of mitotic HeLa cells was performed using both pre- and post-embedding techniques. Mitotic cells were collected by the mitotic shake-off method (Leser et al., 1989). For the pre-embedding method the cells were attached to poly-L-lysine–coated plastic petri dishes and briefly extracted with 0.1% Triton X-100 in CSK buffer (Fey et al., 1986) containing 0.1 mM PMSF, for 30–60 s on ice, and fixed in 3.7% paraformaldehyde in CSK buffer for 30 min at room temperature. After fixation the cells were further extracted with 0.5% Triton in CSK buffer for 30 min, washed three times for 10 min with PBS and rinsed twice with Tris buffer (20 mM Tris-HCl, pH 8.2, 0.5 M NaCl, 0.05% Tween 20, and 0.1% BSA). The cells were incubated with rabbit anti-coilin antibody diluted 1:50 in Tris buffer, for 2 h at room temperature, washed three times for 10 min in the same buffer, and incubated with goat anti-rabbit IgG coupled to 5 nm gold particles (Amersham Corp., Arlington Heights, IL) for 2 h at room temperature. After washing in Tris buffer, the samples were rinsed three times with PBS, fixed in 2% glutaraldehyde in PBS for 10 min, post-fixed in 0.05% $OsO_4$, contrasted with 0.2% tannic acid, and 0.5% uranyl acetate/1% phosphotungstic acid, and embedded in Epon as described by Langanger et al. (1984). For the post-embedding method the cells were fixed in 1% glutaraldehyde in PBS for 30 min at 4°C, dehydrated in ethanol, and embedded in LR White resin (The London Resin Co., Hampshire, U.K.). Ultrathin sections were briefly rinsed in PBS containing 0.05% Tween 20, incubated with the monoclonal antibody 3C5 for 1 h at room temperature, washed in the same buffer, and incubated with a secondary antibody anti-mouse IgM coupled to 10 nm gold particles (Janssen Life Sciences Products, Beerse, Belgium). Sections were then stained with aqueous uranyl acetate and lead citrate. Samples were examined with both a Philips EM 301 and a JEOL 100CXII electron microscope operated at 80 kV.

## Results

### Mitotic Cells Contain at Least Two Distinct Populations of snRNP-containing Structures

In agreement with data from earlier studies (Reuter et al., 1985; Spector and Smith, 1986; Verheijen et al., 1986; Leser et al., 1989; Spector et al., 1991), we observe that cells entering mitosis show a predominantly diffuse snRNP staining pattern that is excluded from condensed chromatin (Fig. 1, *A* and *B*). In addition, we detect a subset of splicing snRNPs concentrating in discrete structures in the mitotic cytoplasm and observe that the fraction of total snRNP concentrating in these structures increases as cells progress through mitosis. These structures are labeled with antibodies directed against both snRNP proteins and the m3G cap structure, and by snRNA-specific antisense probes, indicating that they contain assembled snRNP particles (Figs. 1 and 3; and other data not shown). Similar results were obtained using different fixation/permeabilization methods. In addition to the HeLa cells presented in this study, the presence of snRNP-containing punctate structures in the cytoplasm of mitotic cells was also observed in a variety of other cultured cell lines, including Hep 2, Vero, and MEL (murine erythroleukemia) cells (data not shown).

We addressed next whether the punctate snRNP-containing structures correspond to mitotic forms of either coiled bodies or clusters of interchromatin granules, which both give rise to the characteristic punctate snRNP staining pattern in interphase nuclei (reviewed by Lamond and Carmo-Fonseca, 1993*a*,*b*). Mitotic cells were labeled, therefore, with poly-

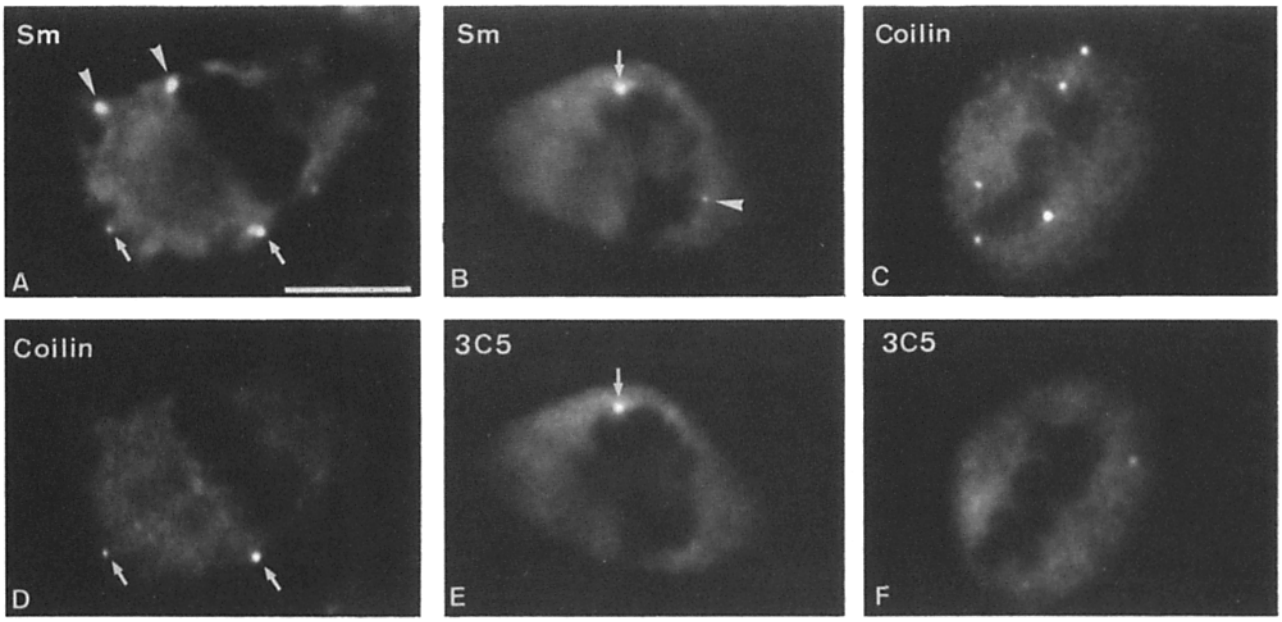


*Figure 1.* snRNPs associate with two distinct structures during mitosis. HeLa cells were double labeled with a monoclonal antibody anti-Sm (*A* and *B*) and either anti-coilin polyclonal antibodies (*D*) or the monoclonal antibody 3C5 (*E*). Some of the structures labeled by anti-Sm antibody are also labeled by anti-coilin antibody (*A* and *D*, *arrows*). However, there are additional Sm-containing structures which are not labeled by anti-coilin antibodies (*A*, *arrowheads*). Double labeling with anti-Sm and 3C5 monoclonal antibodies show that some snRNP-containing structures are labeled by the 3C5 antibody (*B* and *E*, *arrows*); in addition there are Sm-labeled structures that do not colocalize with 3C5 (*B*, *arrowhead*). Cells double labeled with anti-coilin and 3C5 antibodies show that the structures labeled by each of these antibodies do not colocalize (*C* and *F*). Bar, 10 μm.

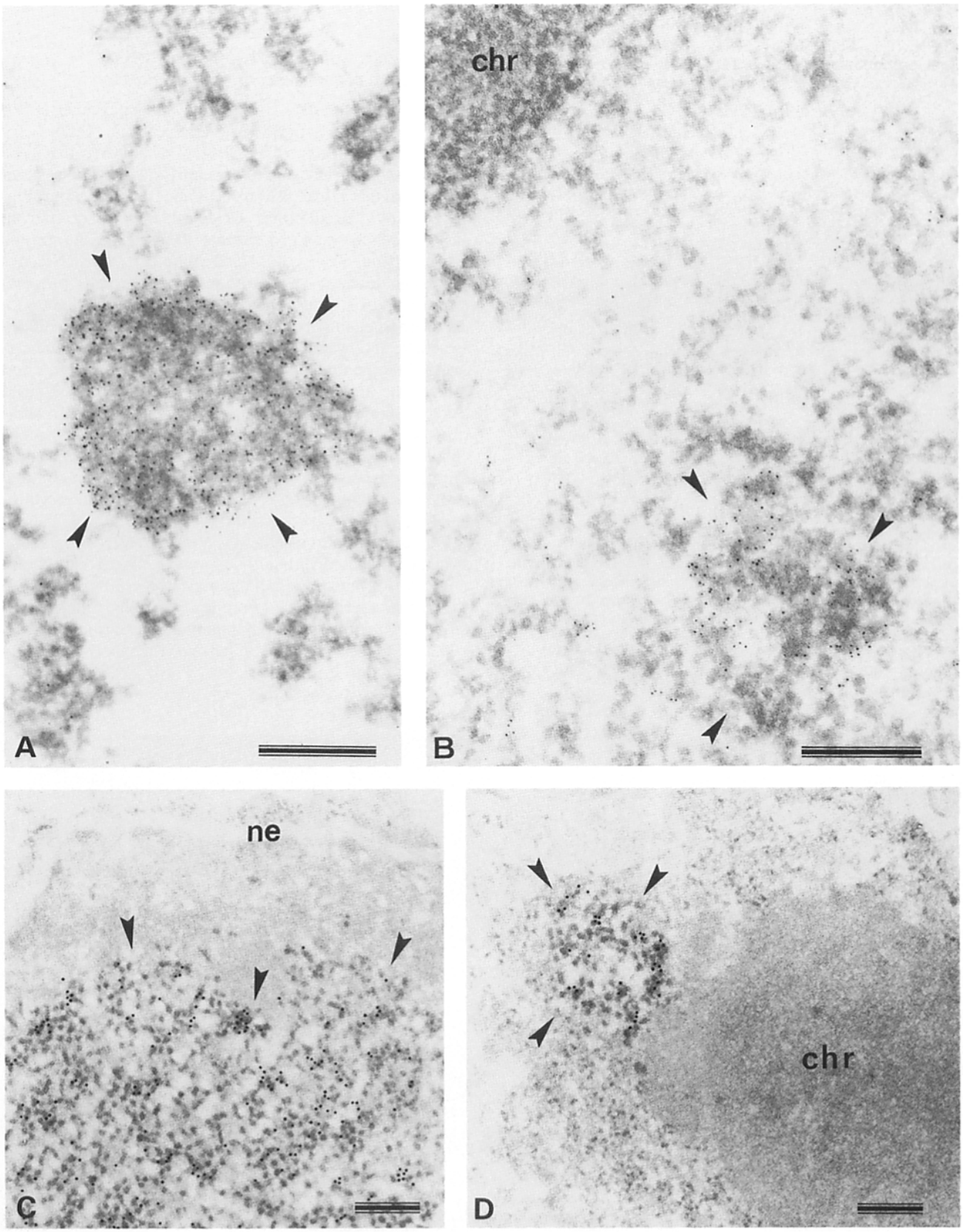


*Figure 2.* Electron microscopy of MCBs and MIGs. Interphase (*A* and *C*) and mitotic (*B* and *D*) cells were immunogold labeled with either anti-coilin antibodies (*A* and *B*) or with the monoclonal antibody 3C5 (*C* and *D*). Immunolabeling with anti-coilin antibodies was performed using a pre-embedding procedure and 5 nm gold particles. The labeling is specifically concentrated over round structures made of dense knotted threads which correspond to coiled bodies (*A* and *B*, *arrowheads*). Immunolabeling with the monoclonal antibody 3C5 was performed using a post-embedding technique and 10-nm gold particles. The immunogold is detected specifically on clusters of interchromatin granules found in the nucleus of interphase cells (*C*, *arrowheads*) and in the cytoplasm of mitotic cells (*D*, *arrowheads*). *Chr*, chromosome; *ne*, nuclear envelope. Bar, 0.25 μm.

clonal antibodies specific for the CB protein, p80 coilin (Andrade et al., 1991) and with the monoclonal antibody 3C5, which predominantly stains interchromatin granules (Turner and Franchi, 1987). Double labeling of mitotic cells with anti-coilin and anti-Sm antibodies clearly shows that both probes label the same foci (Fig. 1, *A* and *D*, *arrows*). However, additional structures are stained by anti-Sm antibodies which are not labeled by anti-coilin antibodies (Fig. 1, *A* and *D*, *arrowheads*). In agreement with the data of Turner and Franchi (1987), the monoclonal antibody 3C5 also stains discrete structures in the cytoplasm of mitotic cells (Fig. 1, *E* and *F*). Double-labeling with the monoclonal 3C5 and anti-Sm antibodies shows that snRNP is present in the structures labeled by the 3C5 monoclonal antibody (Fig. 1, *B* and *E*, *arrow*). We next double-labeled cells with anti-coilin antibodies and the monoclonal antibody 3C5. The structures labeled by each of these antibodies were never seen to colocalize (Fig. 1, *C* and *F*).

To characterize further the structures labeled by the anti-coilin and 3C5 antibodies in mitotic cells, we performed immuno-electron microscopy (Fig. 2). In mitotic cells, the anti-coilin antibodies specifically labeled small structures with a knotted or granular morphology (Fig. 2 *B*), reminiscent of interphase CBs (Fig. 2 *A*). In some cases label was also seen over strands adjacent to the small structures, possibly corresponding to unwound or disassembled "coils" from a coiled body (Fig. 2 *B*). In contrast, the monoclonal antibody 3C5 specifically labeled cytoplasmic clusters of granules in mitotic cells (Fig. 2 *D*), that are similar in appearance to the IGs in the nucleus of interphase cells (Fig. 2 *C*).

In summary, we conclude that, during mitosis, a subset of the total splicing snRNPs associate with at least two, distinct structures; one corresponds to remnants of the coiled body (referred to here as mitotic coiled bodies, MCBs), the other corresponds to clusters of granules that both resemble the EM morphology of interchromatin granule clusters seen in interphase nuclei and are selectively stained by the same monoclonal antibody. We therefore refer to these granular clusters as MIGs (i.e., mitotic interchromatin granules).

### *Kinetics of MCB and MIG Accumulation during Mitosis*

Interestingly, MCBs and MIGs accumulate during mitosis with different kinetics (Table I). Compared to the number of CBs in interphase (on average two per nucleus), the number of MCBs that can be detected is higher (on average five to seven per cell) and remains approximately constant from metaphase to telophase. In contrast, there are ~20–50 IGs per interphase nucleus but only an average of approximately two in metaphase cells. This number can increase dramatically (up to 70–100) as the cells proceed to telophase. The size of individual MIG clusters also become progressively larger in anaphase and telophase cells. Thus MIGs, but not MCBs, proliferate as cells progress through mitosis.

### *U1 and U2 snRNPs Colocalize in MCBs and MIGs*

We next sought to extend the observations that snRNPs are present in MCBs and MIGs by addressing whether different snRNP species colocalize in each structure or whether they contain specifically one type of snRNP. First, we performed double-labeling experiments using anti-snRNP specific protein antibodies and anti-snRNA antisense probes to establish that both RNA and protein components, i.e., assembled snRNP particles, were present. The labeling patterns of both an anti-U1 snRNA oligonucleotide and a monoclonal antibody directed against the U1snRNP-specific 70K protein colocalized precisely in mitotic cells (Fig. 3, *A* and *D*). Similarly, the labeling patterns of an anti-U2 snRNA oligonucleotide probe and a monoclonal antibody specific for the U2 snRNP B″ protein also colocalized (Fig. 3, *B* and *E*). These data indicate that assembled snRNPs are being detected. To address whether U1 and U2 snRNPs colocalize in the same, or different, structures, cells were double labeled with an anti-U1 snRNA probe and a monoclonal antibody specific for the U2 snRNP protein B″. The same mitotic structures are stained by both probes (Fig. 3, *C* and *F*). Double labeling of cells with anti-U1 and U2 snRNP probes, and with anti-coilin antibodies and the monoclonal antibody 3C5, reveals that each of the spliceosomal snRNPs colocalize both in MCBs and MIGs (data not shown).

In summary, we conclude that there are not separate mitotic structures for each type of snRNP particle and that assembled snRNPs colocalize in both MCBs and MIGs.

*Table I.*

| | CBs/MCBs | IGs/MIGs |
|---|---|---|
| Interphase | 2.02 (1.16) | 25.24 (3.98) |
| Metaphase | 7.68 (5.54) | 1.74 (2.56) |
| Anaphase | 5.22 (3.62) | 22.88 (21.67) |
| Telophase | 5.36 (4.14) | 70.60 (25.73) |

The mean number of MCBs and MIGs throughout mitosis is shown with the standard deviation indicated in parentheses. Each mean is calculated from a sample size of 30-60 HeLa cells, labeled with either anti-coilin antibodies (MCB) or mAb 3C5 (MIG). The number of structures stained by each antibody in a given cell was counted in serial optical sections using a confocal fluorescence microscope. A paired *t*-test was applied to compare the mean number of structures present at each stage of the cell cycle. The mean number of MCBs does not significantly differ from anaphase to telophase ($P = 0.89$). In contrast, the mean number of MIGs varies significantly from metaphase to anaphase and from anaphase to telophase ($P = 0.001$). Compared to CBs and IGs in interphase, the mean number of MCBs and MIGs in metaphase is significantly different ($P = 0.001$).

### *Transcription Recommences during Late Telophase*

An important question concerning the organization of splicing snRNPs during the cell division cycle concerns the timing of import of snRNPs and protein splicing factors into daughter nuclei, relative to the time at which transcription is reactivated. To address this question we have analyzed whether mitotic cells are transcriptionally active using two, recently developed methods which allow in situ visualization of brominated uridine that is incorporated into nascent RNA chains (Jackson et al., 1993; Wansink et al., 1993) (Fig. 4). In agreement with these previous studies, we observe the sites of RNA synthesis in interphase nuclei appearing as hundreds of small foci, widely spread throughout the nucleoplasm (Fig. 4, *A* and *B*). The detection of nascent transcription sites is totally inhibited in the presence of α-amanitin (Fig. 4 *C*; and other data not shown). In prometaphase, metaphase, and anaphase, we do not detect any incorporation of Br-UTP (Fig. 4, *D–I*), confirming that transcription ceases during mitosis (Prescott and Bender, 1962). At early

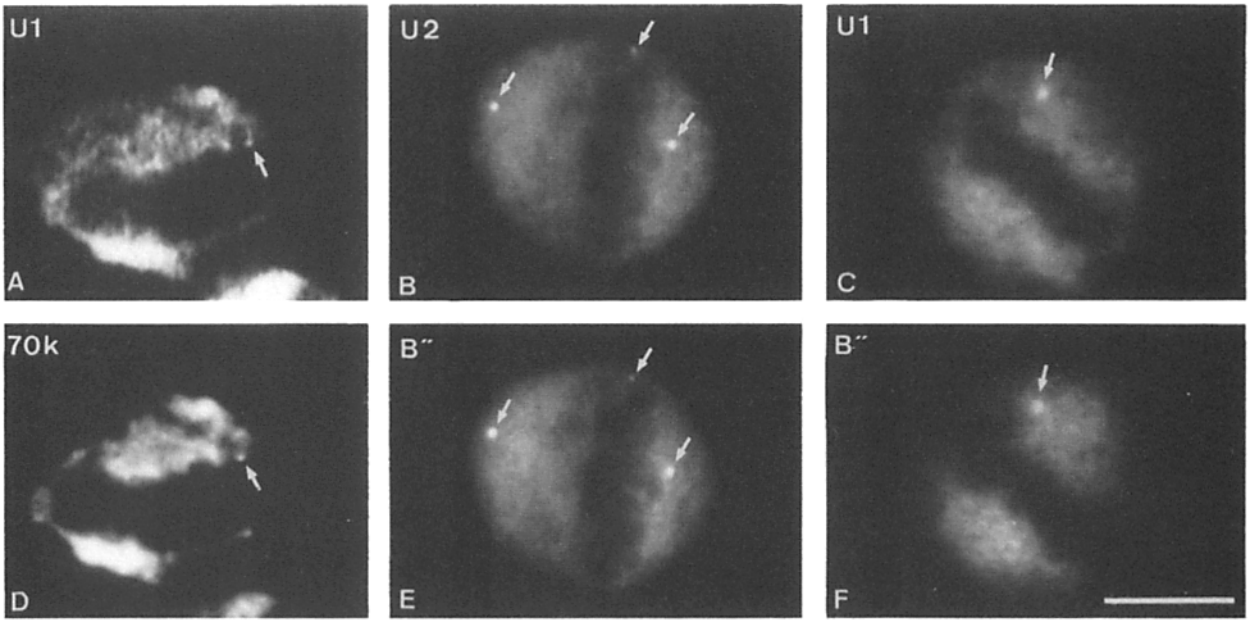


*Figure 3.* In mitotic cells U1 and U2 snRNAs co-localize with cognate snRNP proteins. HeLa cells were double labeled with a U1snRNA specific antisense probe (*A*) and a monoclonal antibody directed against the U1snRNP-specific 70K protein (*D*). Both probes label the same discrete structures in the cytoplasm (*arrow*). Similarly, an antisense probe specific for the U2snRNA (*B*) and a monoclonal antibody against the U2snRNP-specific B″ protein (*E*) are shown to colocalize (*arrows*). Double labeling with the anti-U1snRNA probe (*C*) and the anti-B″ monoclonal (*F*) also show a perfect colocalization (*arrow*), indicating that U1 and U2snRNPs co-localize in the mitotic structures. Bar, 10 μm.

telophase, when the nuclear envelope re-forms and the chromosomes start to decondense, we also detect no transcriptional activity (Fig. 4, *J* and *M* and *K* and *N*, *arrowheads*). In contrast, the nuclei of late telophase cells are clearly transcriptionally active (Fig. 4, *J* and *M* and *L* and *O*, *arrows*). When observed by phase contrast light microscopy, the transcriptionally active nuclei appear spheroidal, the chromatin is decondensed and nucleoli are often visible (Fig. 4, *J* and *L*, *arrows*).

In summary, the data show that transcription is off during mitosis and specifically recommences when cells reach late telophase.

### *The Nuclear Import of Spliceosomal snRNPs Correlates with the Onset of Transcription in Daughter Nuclei*

Having established that transcription reinitiates at late telophase, we next addressed the question of whether snRNP transport into daughter nuclei occurs prior to, in parallel with, or lags behind the onset of transcription. Therefore, double-labeling experiments were performed to visualize both the incorporation of Br-UTP and the localization of snRNP antigens (Fig. 5). In more than 100 telophase cells examined, snRNP staining was always detected within transcriptionally active nuclei (Fig. 5, *A–C*). All telophase nuclei that incorporated Br-UTP were also labeled by anti-Sm antibodies, indicating that snRNP import cannot lag behind the reactivation of transcription. The data are consistent with nuclear import of splicing snRNPs taking place at approximately the same time as the reactivation of transcription in daughter nuclei.

### *MIGs Lacking snRNPs Persist in the Cytoplasm of Transcriptionally Active Cells*

Since the splicing snRNPs increasingly concentrate in MIGs during late stages of mitosis, we have studied the timing of import of MIG antigens relative to the re-entry of snRNPs and the onset of transcriptional activity in daughter nuclei. Double-labeling experiments, using Br-UTP incorporation and the monoclonal antibody 3C5, revealed that MIGs are still present in the cytoplasm of transcriptionally active telophase cells (Fig. 5, *D–F*). Interestingly, however, MIGs persist in the cytoplasm of transcriptionally active cells but no longer contain splicing snRNPs (Fig. 6). This implies that snRNPs leave MIGs to re-enter the nucleus. MIG antigens are subsequently detected in the nucleus at very late telophase/early G1. As cells progress through G1, the characteristic speckled pattern reappears and snRNPs are again found in association with clusters of IGs (Fig. 6, *E* and *F*).

### *Separate Components of the Splicing Machinery Are Asynchronously Imported into Daughter Nuclei*

At the end of mitosis nuclear components are reimported into daughter nuclei, but not all molecules are transported simultaneously. For example, it has been shown recently that hnRNP C proteins are transported into the nucleus before hnRNP A1 proteins (Piñol-Roma and Dreyfuss, 1991). We have, therefore, compared the timing of nuclear import of different components of the splicing machinery (Fig. 7). Splicing snRNPs are imported before hnRNP A proteins (Fig. 7, *A* and *B*). In contrast, MIGs remain in the cytoplasm even when the hnRNP A proteins have already been im-

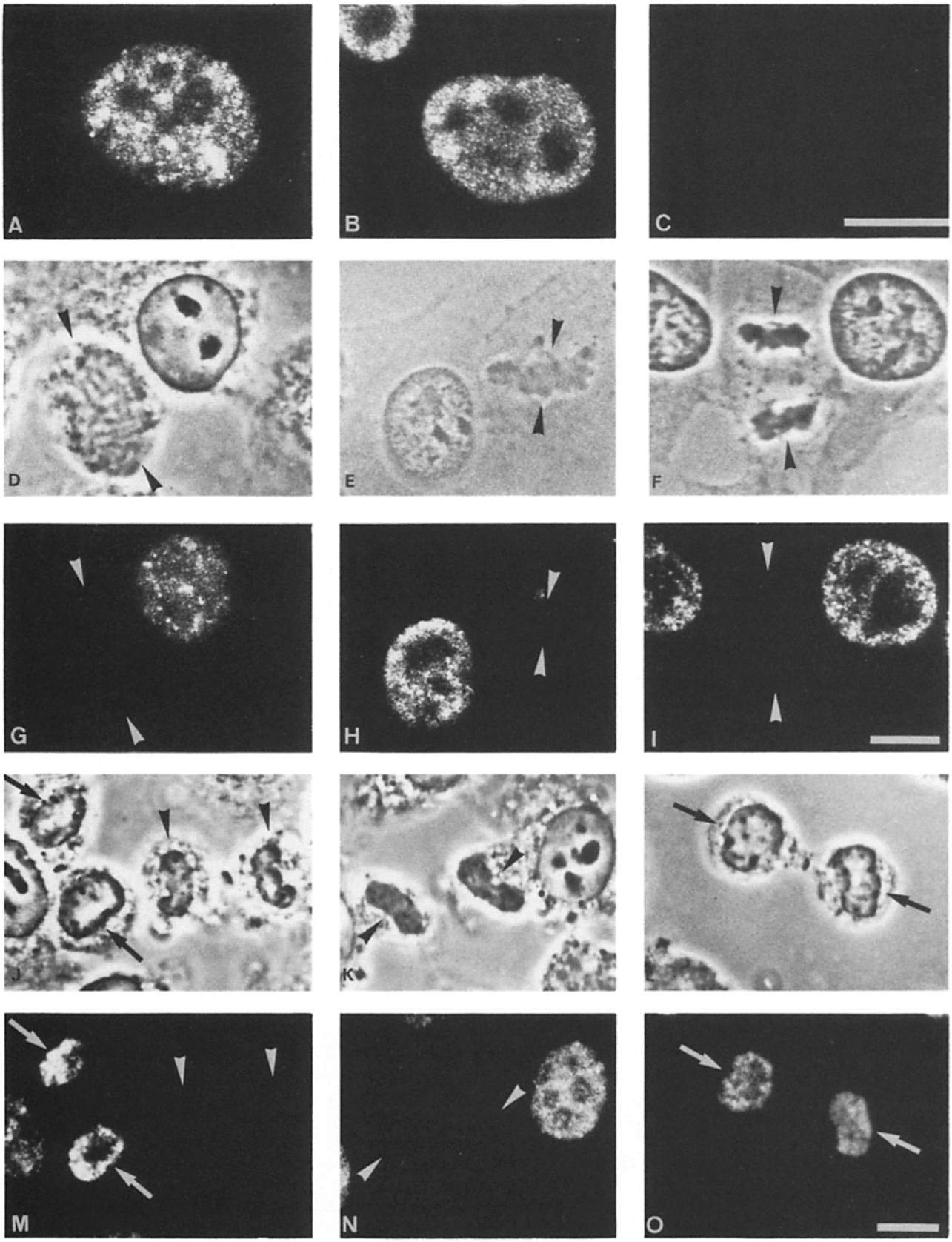


*Figure 4.* In situ visualization of transcription sites during mitosis. Nascent transcripts were visualized in both HeLa (*A*, *D*, *G*, and *J–O*) and Hep-2 (*B*, *C*, *E*, *H*, *F*, and *I*). In interphase cells the sites of transcription are visualized as multiple foci distributed throughout the nucleoplasm (*A* and *B*). Addition of α-amanitin (50 μg/ml) during the transcription reaction abolishes labeling (*C*). In prometaphase (*D* and *G*), metaphase (*E* and *H*), and anaphase (*F* and *I*) no labeling is detected over the chromosomes (*arrowheads*). During early telophase (*J* and *M*, *arrows*, and *K* and *N*, *arrowheads*) when the chromatin is still mostly condensed, as assessed by phase contrast microscopy, no labeling is detected. In contrast, late telophase cells (*J* and *M*, *L* and *O*, *arrows*) are intensely labeled. Bar, 10 μm.

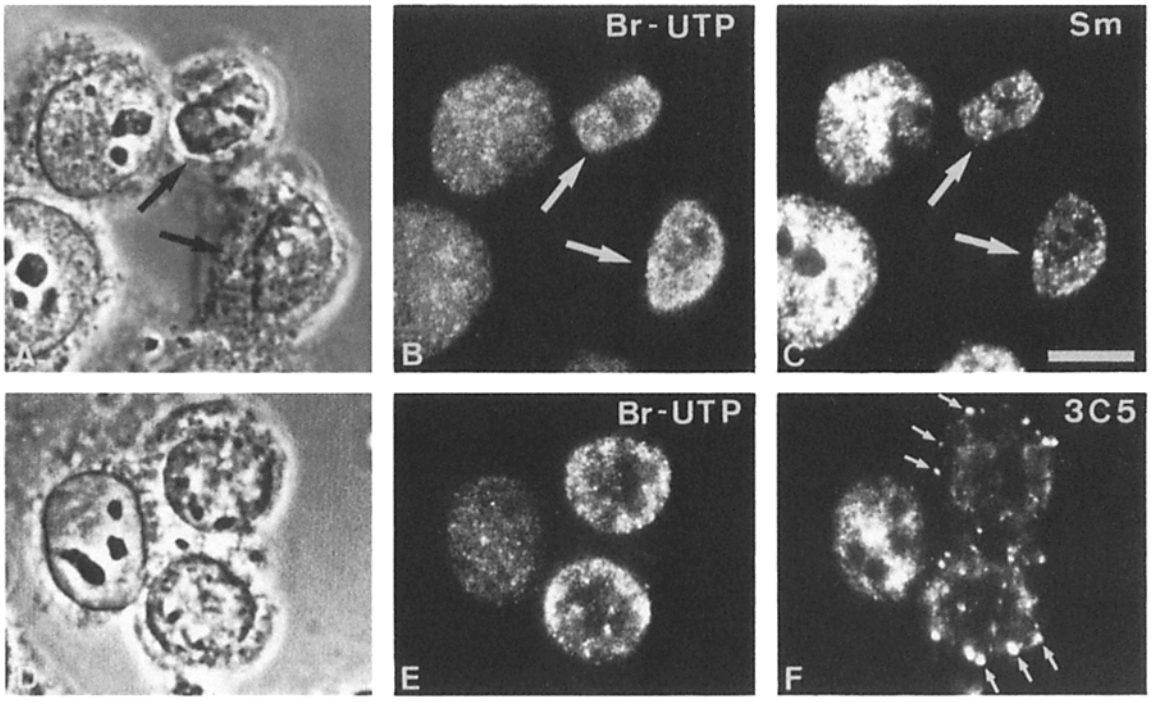


*Figure 5.* Transport of snRNPs into daughter nuclei correlates with the onset of transcription. HeLa cells were permeabilized and incubated with Br-UTP in order to visualize the sites of transcription (*B* and *E*). The same cells were then immunolabeled with either anti-Sm (*C*) or 3C5 antibodies (*F*). Sm staining is detected within the nuclei of transcriptionally active telophase cells (*A–C, arrows*). In contrast, the 3C5 monoclonal antibody labels numerous large structures that remain in the cytoplasm of transcriptionally active cells (*D–F, arrows*); in addition to these large cytoplasmic clusters the monoclonal 3C5 shows a faint nucleoplasmic staining. Bar, 10 μm.

ported into the nucleus (Fig. 7, *C* and *D*). The splicing factor SC-35 has also been observed to remain clustered in the cytoplasm of telophase cells after snRNPs have reentered the nucleus (Spector et al., 1991). We observe a similar effect and show that SC-35 remains concentrated in cytoplasmic MIGs, as determined by double labeling with the monoclonal antibody 3C5 (Fig. 7, *E* and *F*, and data not shown). However, both 3C5 and anti-SC-35 mAbs also show a faint, widespread nucleoplasmic staining in addition to the cytoplasmic granules. The data indicate that SC-35 can remain associated with cytoplasmic MIGs even after snRNPs and hnRNP proteins C and A have been imported to the nucleus. This is surprising, since transcription is highly active in these cells, as demonstrated by double-labeling experiments using incorporation of Br-UTP and the monoclonal antibody 3C5 (Fig. 5, *D–F*). Since in vitro data indicate that SC-35 is an essential splicing factor (Fu and Maniatis, 1990), the results may imply that a subset of SC-35 left MIGs and entered the nucleus, hence explaining the faint nucleoplasmic labeling seen at this stage. Alternatively, it may be that the nascent RNA produced in late telophase cells is not being spliced or that some splicing may take place in vivo via an SC-35 independent mechanism. Further experiments are needed to address these possibilities.

CBs are not observed in most telophase cells. They form later during G1, after a lag period which is variable from cell to cell (Andrade et al., 1993). However, anti-coilin antibodies reveal a faint, widespread nucleoplasmic staining of telophase nuclei, indicating that coilin has been imported but CBs have not yet assembled (data not shown; see also Fig. 8 *D*).

### *Transport of Spliceosomal Components into Daughter Nuclei Does Not Require Ongoing Transcription*

To extend the analysis of how spliceosomal components are imported into the daughter nuclei, we tested the effect of RNA polymerase inhibitors on the transport of snRNPs, MIG antigens, the splicing factor SC-35 and coilin (Fig. 8). HeLa cells were treated for 45–50 min with actinomycin D and the inhibition of transcription confirmed by analysis of Br-UTP incorporation (data not shown). In agreement with the data of Piñol-Roma and Dreyfuss (1991), actinomycin D blocked the nuclear import of hnRNP A1 protein (Fig. 8, *A*, *C*, *E* and *G*). Similar results were observed in cells treated with 5,6-dichlorobenzimidazole riboside, another inhibitor of RNA polymerase II, but not in cells treated with actinomycin at a concentration that does not inhibit RNA polymerase II (data not shown; Piñol-Roma and Dreyfuss, 1991). However, inhibition of RNA polymerase II did not prevent the nuclear import of snRNPs (Fig. 8 *B*), in agreement with previous data from Zieve and Slitzky (1986). Inhibition of transcription also did not prevent the nuclear import of coilin and proteins associated with MIGs, including the splicing factor SC-35 (Fig. 8, *A–H*). Nor did it block the association of splicing snRNPs with IGs in daughter nuclei (Fig. 8, *I* and *J*). However, formation of snRNP-containing CBs was not observed (Fig. 8; and other data not shown).

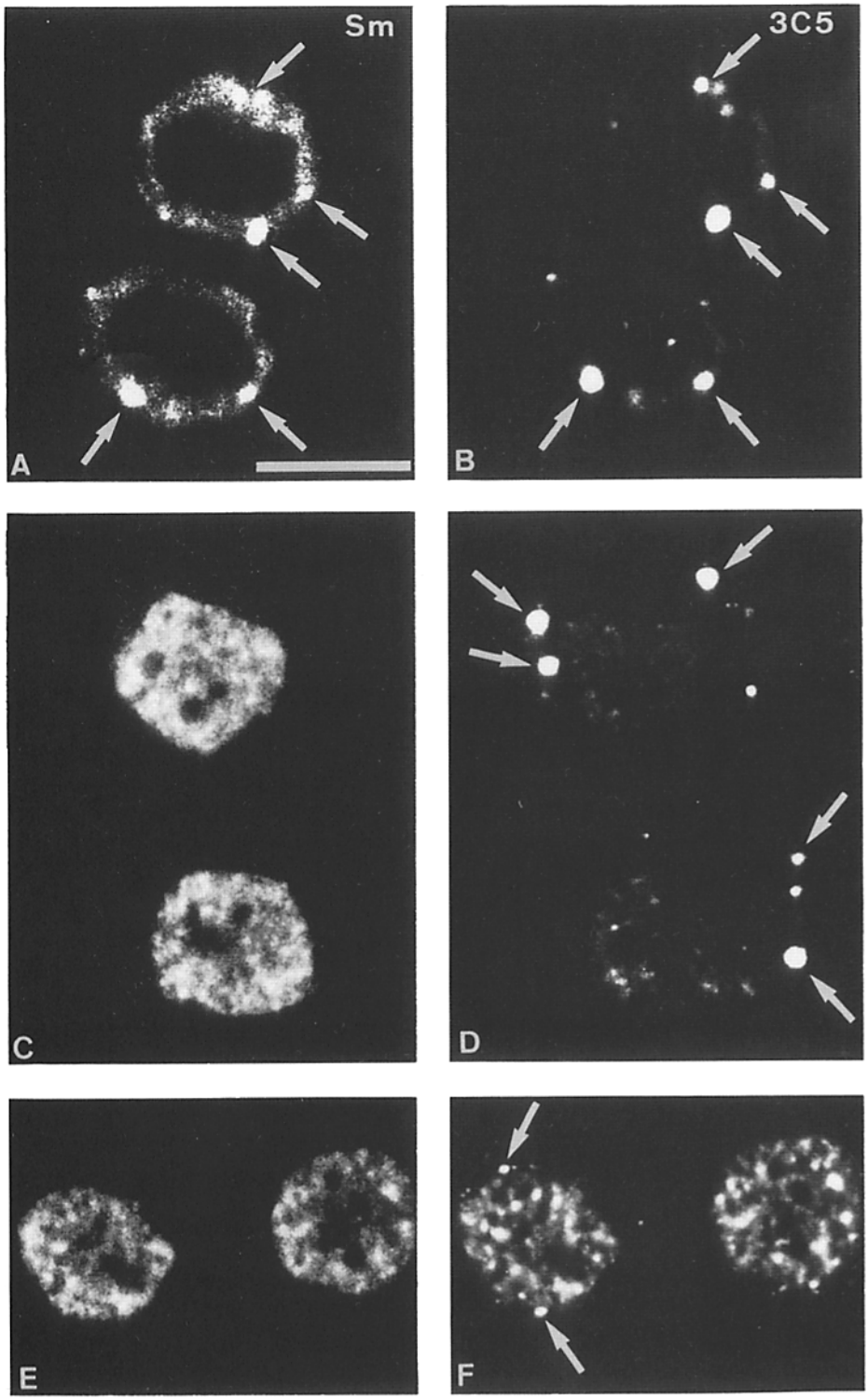


*Figure 6.* snRNPs enter telophase nuclei before MIG antigens. HeLa cells were double labeled with anti-Sm (*A*, *C*, and *E*) and 3C5 (*B*, *D*, and *F*) antibodies. In early telophase cells the Sm staining is diffuse in the cytoplasm and in addition concentrates in discrete structures that colocalize with MIGs labeled by the monoclonal antibody 3C5 (*A* and *B*, *arrows*). In late telophase cells Sm is exclusively detected in the nucleus (*C*), while MIGs labeled by 3C5 persist in the cytoplasm (*D*, *arrows*). In G1 cells the 3C5 monoclonal antibody labels intranuclear speckles (i.e., IGs) that are also labeled by anti-Sm antibodies (cf., *E* and *F*); however, a few MIGs are still detected in the cytoplasm (*F*, *arrows*).

In summary, the results demonstrate that both the nuclear targeting of snRNPs and their subsequent interaction with clusters of IGs following mitosis are not dependent upon ongoing transcription.

## *Discussion*

In this study, we show that a subset of splicing snRNPs are present in distinct structures in the cytoplasm of mitotic cells. These structures correspond to mitotic forms of CBs and clusters of IGs, which we refer to here as MCBs and MIGs, respectively. Both U1 and U2 snRNPs are detected in the same MCBs and MIGs throughout mitosis, indicating that separate splicing snRNPs are not partitioned between these structures. A similar situation occurs in interphase, where each of the splicing snRNPs is found both in nuclear CBs and IGs. While only a small fraction of the total snRNP associates with MCBs and MIGs during the early stages of

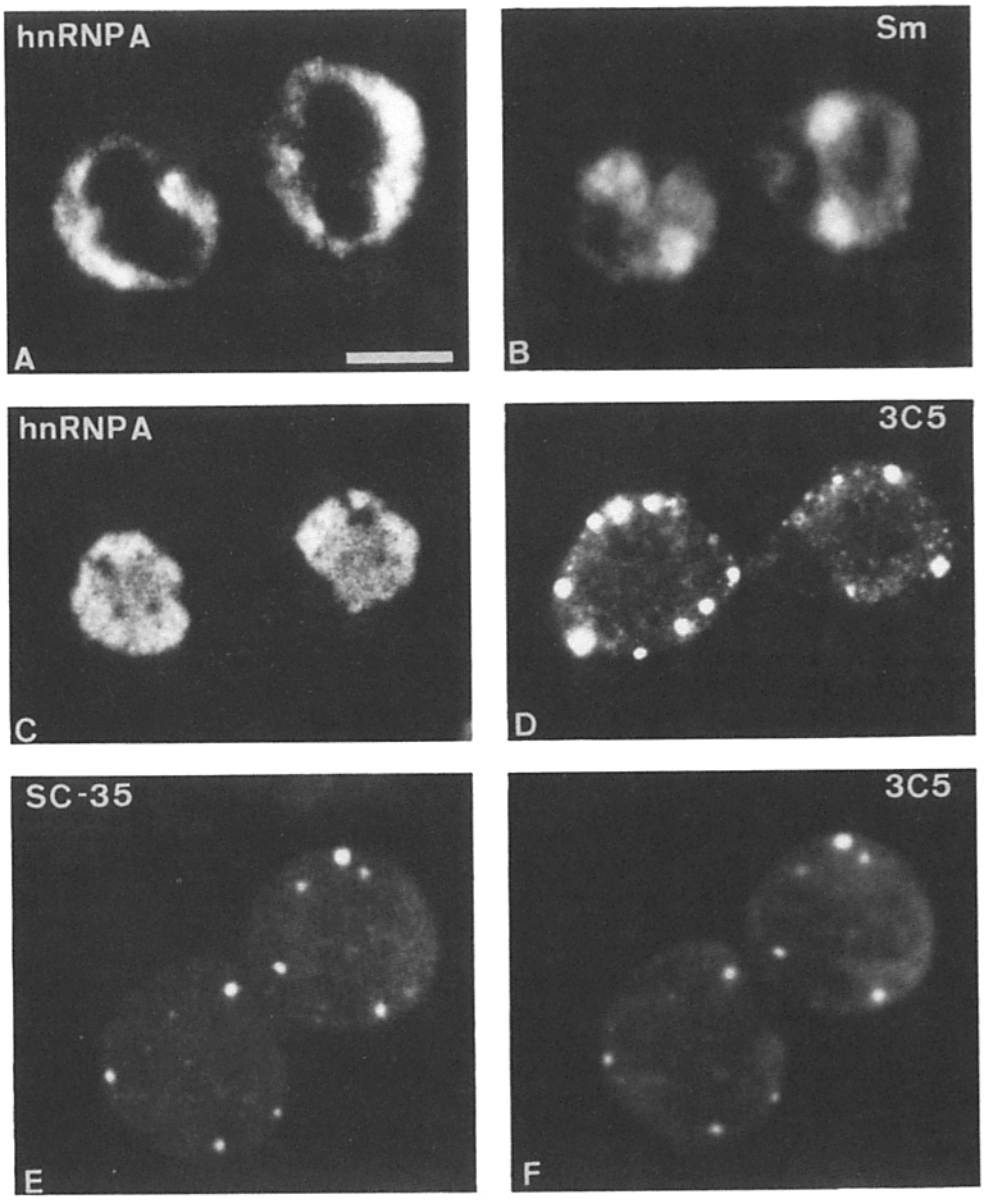


*Figure 7.* Asynchronous import of splicing components into daughter nuclei. Telophase HeLa cells were double labeled with monoclonal 4B10 (specific for the hnRNP A proteins) and antibodies directed against different components of the splicing machinery. In telophase cells, where hnRNPA is exclusively detected in the cytoplasm (*A*), anti-Sm antibodies already reveal strong nucleoplasmic staining (*B*). In contrast, in cells where the hnRNP A protein is exclusively detected within the nucleus (*C*), and the monoclonal antibody 3C5 predominantly labels large cytoplasmic structures (*D*). In order to correlate the timing of import of hnRNP A proteins with that of the splicing factor SC-35, cells were double labeled with anti-SC-35 and 3C5 monoclonal antibodies. Both these antibodies show a perfect colocalization (*E* and *F*). Bar, 10 μm.

the cell division cycle, we observe a dramatic proliferation of snRNP-containing MIGs as cells progress into telophase. In contrast, the MCBs remain approximately constant in number and size and contain only a small proportion of snRNP throughout mitosis. This suggests that MCBs and MIGs have different roles, as previously proposed for interphase CBs and IGs (reviewed in Lamond and Carmo-Fonseca, 1993*a*,*b*).

The dramatic accumulation of snRNPs in MIGs, but not in MCBs, as cells progress through mitosis indicates that snRNP recruitment into MIGs is not dependent upon the presence of actively transcribed genes within (or linked to) these structures. Similarly, in interphase cells snRNPs accumulate in IGs in the absence of active gene expression (Carmo-Fonseca et al., 1992; Antoniou et al., 1993). At late stages of erythroid differentiation in vitro, when the cells enucleate, snRNPs become concentrated in very large cytoplasmic clusters of IGs (Antoniou et al., 1993), reminiscent of the situation in mitotic cells. It has also been observed that snRNPs accumulate in IG clusters in cells infected with herpes simplex virus, where the predominant transcription is of viral genes that do not contain introns and, hence, are not spliced (Martin et al., 1987; Phelan et al., 1993). In combination, these observations show that the presence of snRNPs in interchromatin granules is not obligatorily coupled to splicing. Instead, the recruitment of snRNPs into MIGs at telophase suggests that these structures may have a role in preassembly of snRNPs, and possibly other components of the splicing machinery, allowing them to participate in the splicing of nascent transcripts once they enter the nucleus of daughter cells. The data cited above are consistent with nuclear interchromatin granule clusters playing a similar role in snRNP assembly during interphase. However, this does not exclude that IGs may also play additional roles connected with other steps in the RNA processing pathway. For example, they may act as storage structures, since snRNPs can ac-

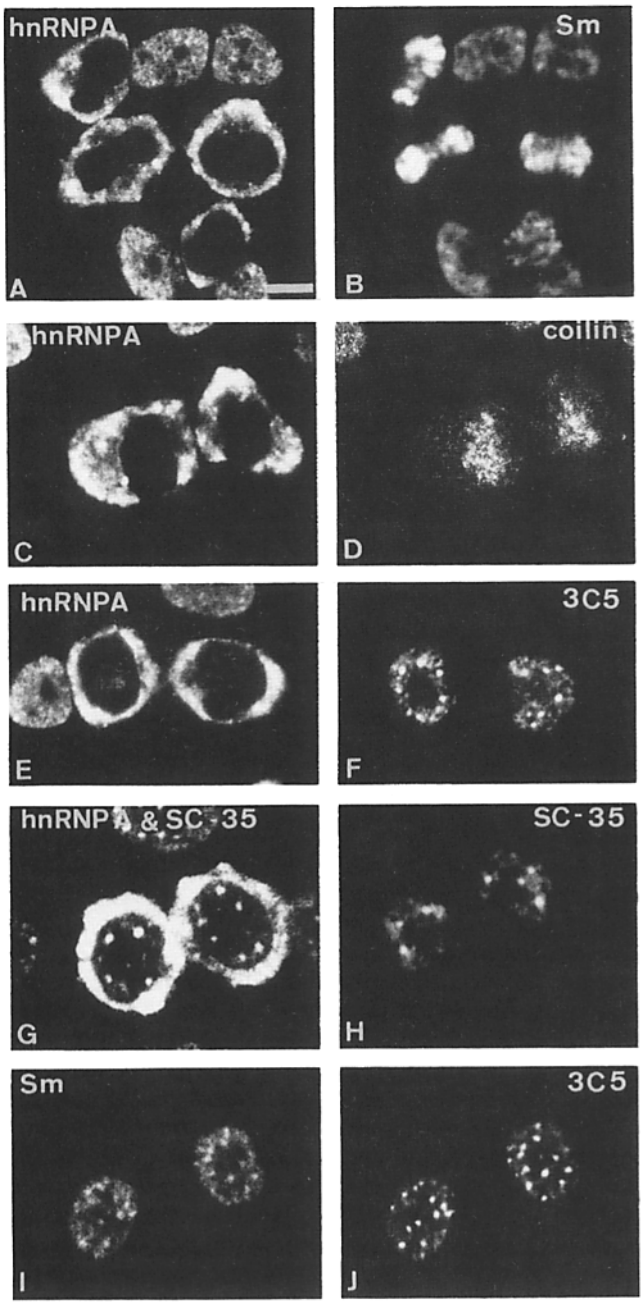


*Figure 8.* Actinomycin D does not prevent the import of spliceosomal components into daughter nuclei. HeLa cells were treated with actinomycin D (5 μg/ml) for 45–50 min and immunolabeled with the monoclonal antibody 4B10 (*A*, *C*, and *E*). Double labeling of the same cells with anti-Sm (*B*), anti-coilin (*D*), and 3C5 (*F*) antibodies show that all these antigens are imported into the nucleus whereas hnRNP A protein staining remains exclusively in the cytoplasm. The splicing factor SC-35 is also detected in the nucleus of actinomycin D–treated cells (*H*). Simultaneous labeling with 4B10 and anti-SC-35 antibodies reveals the typical intranuclear "speckles" labeled by anti-SC-35 whereas the hnRNP A protein remains in the cytoplasm (*G*). Double labeling of actinomycin D–treated cells with anti-Sm and 3C5 monoclonal antibodies shows that snRNPs associate with the nuclear "speckles" labeled by mAb 3C5, which correspond to clusters of interchromatin granules (*I* and *J*, *arrows*).

cumulate there in the absence of RNA synthesis. The localization of poly(A) RNA in, or adjacent to, these structures is also consistent with their having a more direct role in splicing (Carter et al., 1993; Xing et al., 1993; Visa et al., 1993*b*).

In contrast with IGs, which are clearly present in the nucleus of cells at late telophase/G1, CBs are generally not visible in early G1 nuclei (Andrade et al., 1993). Since there is evidence that splicing occurs co-transcriptionally (Beyer and Osheim, 1988, 1991; LeMaire and Thummel, 1990) and we show here that transcription resumes during telophase, this argues against CBs being major sites of splicing. Rather, these results are consistent with our previous interpretation that CBs may be involved in post-splicing events, e.g., recycling of snRNPs or intron degradation (Carmo-Fonseca et al., 1992, 1993; Lamond and Carmo-Fonseca, 1993*b*). According to this model, the assembly of CBs after mitosis could be triggered by, for example, the accumulation of snRNPs in post-splicing complexes or the generation of spliced introns. This would explain why they form only after a lag period following the commencement of transcription. A post-splicing function would also account for the observation that snRNPs do not show any increased association with MCBs during telophase, since no new splicing substrates are transcribed during mitosis. An alternative possibility is that CBs may play a more direct role in splicing, but represent specialized structures for the processing of specific pre-mRNAs which are differentially expressed during the cell cycle. Since these possibilities are not mutually exclusive, the CB could also play more than one role in processes connected with snRNP structure or function.

Recently, it has been shown that CBs are dynamic nuclear organelles that change during the cell cycle (Andrade et al., 1993; Carmo-Fonseca et al., 1993). In this study we show that in metaphase, anaphase, and early telophase cells, anti-coilin antibodies show a widespread staining of the cytoplasm, but also label a few bright foci that are usually smaller than interphase CBs (Fig 1 *C*; and see Carmo-Fonseca et al., 1993). Andrade et al. (1993) also reported widespread coilin staining in the mitotic cytoplasm, but did not observe any small foci remaining. Apart from this minor discrepancy, all the data suggest that CBs predominantly disassemble during mitosis, when no new transcription occurs. Whatever the precise function of the CB may be, this observation, and the related finding that reappearance of CBs in daughter nuclei is transcription-dependent, supports the view that the metabolic role(s) of the CB is connected in some way with active gene expression.

By using recently developed methods that allow the in situ visualization of transcription sites (Jackson et al., 1993; Wansink et al., 1993), we were able to distinguish two functional stages of telophase. During the early stage, there is no transcription and snRNPs are present in the cytoplasm. At the late stage, transcription starts and snRNPs enter the nucleus. This indicates that, as new transcripts are synthesized in the nucleus, snRNPs are already available to initiate splicing. It is surprising, therefore, that the essential splicing factor, SC-35, remains predominantly in the cytoplasm of transcriptionally active telophase cells. One possible explanation is that at least a subset of protein splicing factors are actually present in the nucleus at this stage, but are not efficiently detected by antibodies. Consistent with this, we observe a faint,

widespread nucleoplasmic staining with both the anti-SC-35 and 3C5 mAbs in transcriptionally active telophase cells. It is therefore important to bear in mind that the normal immunofluorescence-labeling pattern seen with these antibodies in interphase cells may be revealing only part of their overall nuclear distribution. An alternative possibility is that the nascent transcripts synthesized in telophase cells may accumulate unspliced in the nucleus until the protein splicing factors are subsequently imported from the cytoplasm. This will be an important point to address experimentally in future studies.

The sequential entry into the nucleus of snRNPs, hnRNP A, and the splicing factor SC-35 suggests the presence of multiple import signals during telophase that may be coordinated by a common mechanism. It will be important to clarify whether this is a mitotic-specific process, or part of a general transport mechanism that operates throughout the cell cycle. For example, in interphase cells novel snRNP particles are assembled in the cytoplasm and are then targeted to the nucleus by a complex signal which involves both the trimethylguanosine cap structure of the UsnRNAs and the Sm proteins (for a recent review see Mattaj et al., 1993). Since the RNA and protein components of snRNP particles do not dissociate during mitosis (Reuter et al., 1985), it is likely that pre-existing snRNPs are imported into daughter nuclei at the completion of mitosis by a mechanism similar to that of nuclear transport of newly synthesized snRNPs during interphase. It has also been shown that during interphase the hnRNP A protein shuttles between the nucleus and cytoplasm in a transcription-dependent fashion (Piñol-Roma et al., 1991). It will be interesting to establish what molecular mechanisms are involved in the nuclear import signals acting during telophase and how these mechanisms relate to nucleo-cytoplasmic transport events during interphase. Analysis of these transport mechanisms may also explain why the translocation to the nucleus of separate RNA processing factors is asynchronous.

A major conclusion from the present study is that the coiled body and interchromatin granule structures with which splicing snRNPs interact are differentially regulated during the cell division cycle. The data support the view that these structures play distinct roles connected with splicing snRNPs and that they probably also act at different stages of snRNP assembly/disassembly, transport, and/or function. Future experiments aimed at refining our picture of how RNA processing is coordinated with cell division should help to clarify how metabolic processes involved in gene expression may be localized within specific subnuclear structures.

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