# Nuclear Organization of Splicing snRNPs during Differentiation of Murine Erythroleukemia Cells In Vitro

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*Abstract.* Murine erythroleukemia (MEL) cells are erythroid progenitors that can be induced to undergo terminal erythroid differentiation in culture. We have used MEL cells here as a model system to study the nuclear organization of splicing snRNPs during the physiological changes in gene expression which accompany differentiation. In uninduced MEL cells, snRNPs are widely distributed throughout the nucleoplasm and show an elevated concentration in coiled bodies. Within the first two days after induction of terminal erythroid differentiation, the pattern of gene expression changes, erythroid-specific transcription is activated and transcription of many other genes is repressed. During this early stage splicing snRNPs remain widely distributed through the nucleoplasm and continue to associate with coiled bodies. At later stages of differentiation (four to six days), when total transcription levels have greatly decreased, splicing snRNPs are redistributed. By six days postinduction snRNPs were concentrated in large clusters of interchromatin granules and no longer associated with coiled bodies. At the end-point of erythroid differentiation, just before enucleation, we observe a dramatic segregation of splicing snRNPs from the condensed chromatin. Analysis by EM shows that the snRNPs are packaged into a membrane-associated structure at the nuclear periphery which we term the "SCIM" domain (i.e., SnRNP Clusters Inside a Membrane).

In eukaryotic cells extensive posttranscriptional processing of primary transcripts (pre-RNA) is essential for the expression of functional mRNA, tRNA, and rRNA products. These processing events include, base and sugar modifications, exo- and endonucleolytic cleavage, formation of a 3′ terminus of polyadenosine residues (poly A tail) and removal of intervening sequences (splicing). As normally only mature mRNA, tRNA, and rRNA is transported to the cytoplasm, export of RNA from the nucleus must also be a finely regulated process. While extensive progress has been made in recent years in studies of the mechanism of RNA processing reactions in vitro, by comparison much less is known about how these events take place in vivo. There are many interesting questions still to be answered concerning how the various steps connected with the processing and export of RNA are spatially organized within the nucleus and coordinated with transcription.

The major subunits of spliceosomes (i.e., the complexes that catalyze the splicing of nuclear pre-mRNAs), correspond to the U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) (reviewed by Lührmann et al., 1990; Lamond, 1993). The use of autoimmune antisera which recognize protein antigens associated with splicing snRNPs has proven extremely valuable for snRNP localization studies (Lerner and Steitz, 1979; Lerner et al., 1981). More recently, the availability of antibodies raised against purified protein components of both snRNPs, and other protein components of the RNA processing machinery, has added significantly to the range of immunoreagents available for localization studies on splicing and polyadenylation factors (Fu and Maniatis, 1990; Takagaki et al., 1990; Zamore and Green, 1991; Zhang et al., 1992). The organization of splicing snRNPs in situ has largely been studied using indirect immunofluorescence techniques (Northway and Tan, 1972; Deng et al., 1981; Reuter et al., 1984; Spector, 1984; Nymann et al., 1986; Verheijen et al., 1986; Habets et al., 1989) and by immunoelectron microscopy (Fakan et al., 1984; Puvion et al., 1984; Spector, 1990; Visa et al., 1993). The RNA components of splicing snRNPs have been localized by hybridization with antisense oligonucleotide probes made of 2′-O-alkyl RNA (Carmo-Fonseca et al., 1991*a*,*b*, 1992; Huang and Spector, 1992; Matera and Ward, 1993). This can allow the RNA and protein components of individual snRNPs to be detected in parallel by double labeling using a combination of antisense and antibody probes. Individual pre-mRNAs have also been analyzed by in situ hybridization methods. For example, certain transcripts have been identified forming tracks within the nucleus (Lawrence et al., 1989; Huang and Spector, 1991; Xing et al., 1993). These tracks may be reflecting intranuclear transport or export pathways used by mRNAs.

Many studies using the types of reagents described above

have demonstrated that most of the individual components of the RNA processing machinery are concentrated in the nucleus, consistent with the biochemical evidence that major RNA processing events take place before nuclear export. Within the nucleus, splicing snRNPs show a complex localization pattern and associate with several distinct substructures, including coiled bodies and interchromatin granules (reviewed by Lamond and Carmo-Fonseca, 1993*a*,*b*). This raises the possibility that specific steps connected with snRNP function, assembly or transport may take place within dedicated nuclear compartments. EM studies have shown that in *Drosophila* cells snRNPs can bind to, and splice, nascent pre-mRNAs (Beyer and Osheim, 1988). Recent studies involving the in situ colocalization of pre-mRNA, snRNPs, and protein factors required for RNA processing are also starting to give an insight into the spatial distribution of these events in mammalian cells (Carter et al., 1993). However, since most of these studies have been conducted with developmentally stationary cells, any alterations in the distribution of the splicing machinery resulting from changes in the pattern or level of transcription during cellular differentiation still remain to be ascertained.

Murine erythroleukaemia (MEL)[1] cells provide a powerful model system with which to study the nuclear organization of RNA processing reactions. MEL cells are erythroid progenitor cells that have been transformed by the Friend virus complex and can be maintained in culture indefinitely (Friend et al., 1971; Singer et al., 1974). MEL cells can be induced with various chemical agents, such as DMSO, to undergo a program of terminal erythroid differentiation which closely mimics the natural process in vivo. During the course of this differentiation process the MEL cells cease to divide and total transcription decreases such that it is virtually 0 by 6-d postinduction (Sherton and Kabat, 1976; Orkin and Swerdlow, 1977; Patel and Lodish, 1984). During the initial 2–4 d of this process, the expression of erythroid specific genes is induced, in particular those encoding the $\alpha$- and $\beta$-globins, while most other loci are repressed. Thus, MEL cells offer an opportunity to study the nuclear organization of splicing and other RNA processing factors during a physiological differentiation process that results in a dramatic alteration in the transcriptional status of the cell.

In this study we report a detailed analysis of snRNP organization in the nuclei of uninduced MEL cells and during the course of terminal erythroid differentiation. We observe that the nuclear distribution of splicing snRNPs, and their association with specific subnuclear compartments, changes during differentiation in a transcription-dependent fashion.

## Materials and Methods

### Cell Culture

The APRT$^-$ MEL cell line C88 was maintained and induced to undergo erythroid differentiation for up to 6 d in the presence of 2% (vol/vol) DMSO as previously described (Antoniou, 1991). Experiments involving $\alpha$-amanitin (Sigma Chemical Co., St. Louis, MO) were performed with log phase growing cultures (0.5–1 $\times$ $10^6$ cells/ml). Cells were incubated with 50 $\mu$g/ml of $\alpha$-amanitin for 5 h.

1. *Abbreviations used in this paper*: CCM, compact confocal microscope; MEL, murine erythroleukemia; nt, nucleotide.

### In Situ Hybridization and Immunofluorescence

Analysis of cells of in situ hybridization and immunofluorescence was essentially as previously described (Carmo-Fonseca et al., 1992). Briefly, 1–2 $\times$ $10^6$ MEL cells were washed twice in PBS and resuspended in 200 $\mu$l of the same buffer. A 15-$\mu$l aliquot of this cell suspension was then spread over a 1-cm diam coverslip which had been coated with polylysine (Sigma Chemical Co.) by brushing with a 1 mg/ml solution in water and air drying. The cells were allowed to adhere to the coverslip for 1 min and then fixed for 10 min with 3.7% paraformaldehyde in CSK buffer at room temperature as previously described (Carmo-Fonseca et al., 1992). Excess cells, which had not adhered to the coverslip, were moved by periodic swirling of the fixative (1–2-min intervals). The fixed cells were then permeabilized with either 0.5% Triton X-100 in CSK buffer or 0.2% SDS (Carmo-Fonseca et al., 1992). Alternatively, cells were pre-extracted with 0.5% Triton X-100 for 3 min on ice before the paraformaldehyde fixation step.

The antisense 2′-O-allyloligoribonucleotide probes specific for 28S rRNA and U2 snRNA, have been described previously (Carmo-Fonseca et al., 1992). The following mouse mAbs were used: anti-B″ protein ("4G3") (Habets et al., 1989), anti-70K protein (Billings et al., 1982), anti-Sm ("Y12") (Pettersson et al., 1984), and anti-SC-35 splicing factor (Fu and Maniatis, 1990). Polyclonal rabbit antibodies to recombinant p80 coilin (Andrade et al., 1991), anti-peptide antibodies to lamins (Djabali et al., 1991) and affinity-purified goat anti-mouse hemoglobin antibodies (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) were also used. Detection of hybridization sites and immunofluorescence were performed as described (Carmo-Fonseca et al., 1992). Samples were examined using both the EMBL compact confocal microscope (CCM) (Stelzer et al., 1992) and the Zeiss Laser Scanning Microscope LSM 310 (Carl Zeiss, Oberkochen, Germany). The EMBL CCM was operated with excitation wavelengths of 488 (fluorescein fluorescence) and 529 nm (Texas red fluorescence) were selected from an argon-ion laser. The LSM was operated with wavelengths of 488 and 543 nm, selected from an argon-ion and from an helium-neon laser, respectively. For double-labeling experiments each fluorochrome was independently recorded. Pseudo-colored images of both signals were generated and superimposed. Images were photographed on Fujichrome 100 (Fuji Photo Film Co., Ltd., Tokyo, Japan) or Kodak Tmax 100 film (Eastman Kodak Co., Rochester, NY) using a Polaroid Freeze Frame Recorder.

### Electron Microscopy

MEL cells were fixed in suspension in 1.6% glutaraldehyde in 0.1 M Sörensen phosphate buffer, pH 7.3. During the 1-h fixation, the cells were centrifuged at 1,000 or 5,000 *g* for 5 min. The resulting pellets were dehydrated in increasing concentrations of ethanol and embedded in LR White resin (The London Resin Co., Hampshire, UK). Ultra-thin sections were labeled with the mAb 3C5 and a secondary antibody corresponding to anti-mouse IgM coupled to 10-nm gold particles (Janssen Life Sciences Products, Beerse, Belgium). Staining was performed using the EDTA regressive method, which preferentially reveals RNA-containing structures (Bernhard, 1969). Conventional staining consisted of uranyl acetate and lead citrate. Samples were analyzed using an electron microscope. (100CXII; JEOL USA, Peabody, MA).

### Analysis of RNA by S1 Nuclease Protection

Total RNA was extracted from MEL cells by selective precipitation in the presence of 3 M LiCl and 6 M urea as denaturant as previously described (Antoniou, 1991). Assay for selected RNA species was by S1-nuclease protection using end-labeled DNA probes as previously described (Antoniou et al., 1993). The 5′ histone H4 probe is a 460-bp EcoRI-TthIII fragment giving a 180 nucleotide (nt) S1-protected product. The $\beta$ major globin probe is a 700-bp HindIII-NcoI fragment which protects 96 nt from the 5′ region of exon II of the mRNA. The 5′ U6 probe is a 326-bp EaeI-FokI fragment obtained from the *Xenopus* U6 snRNA gene (Krol et al., 1987). This gives an 87-nt full-length S1-protected product from the equivalent mouse sequence. A single base mismatch at +8 between the *Xenopus* mouse U6 snRNAs also gives a lower level of a 79-nt product. Reaction products were resolved on 6% polyacrylamide gels in the presence of 6 M urea as denaturant and autoradiographed with Fuji medical x-ray film, with an intensifying screen, at −70°C.

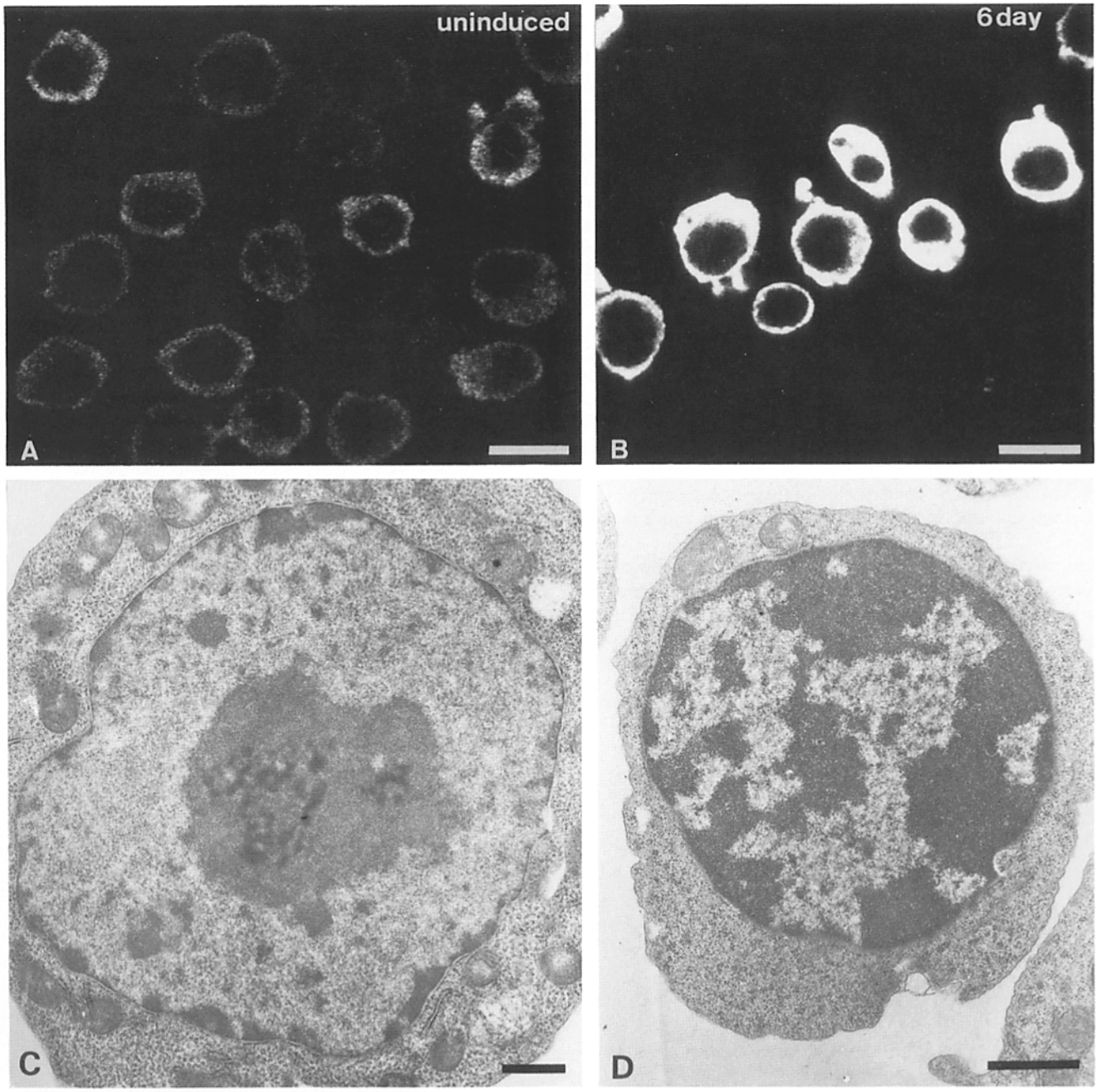


*Figure 1.* Induction of MEL cell differentiation. Uninduced MEL cells (*A* and *C*), and cells at 6 d after terminal erythroid differentiation was induced by DMSO treatment (*B* and *D*), were analyzed by indirect immunofluorescence microscopy using anti-globin antibodies (*A* and *B*) and by EM (*C* and *D*). Bars (*A* and *B*), 10 μm; and (*C* and *D*) 1 μm.

## Results

### Erythroid Differentiation of MEL Cells

The efficient induction of terminal erythroid differentiation upon treatment of MEL cells with medium containing 2% DMSO has been reported in many previous studies (reviewed by Antoniou, 1991). Nonetheless, a series of control experiments were carried out to confirm that we were obtaining a similar high efficiency of induction with the MEL cell line used in the present study. First, a goat anti-mouse hemoglobin antibody was used to detect the levels of globin protein expressed by the uninduced MEL cells as compared with cells at six days postinduction (Fig. 1, *A* and *B*). The anti-mouse hemoglobin antibody showed only very weak fluorescence in the uninduced cells, with <5% of the cells showing evidence of spontaneous induction (Fig. 1 *A*). In contrast, almost all the cells (>95%) showed strong cytoplasmic fluorescence when analyzed using the same an-

tibody at 6-d postinduction (Fig. 1 *B*). Second, EM was performed to compare the morphology of the uninduced MEL cells with cells at 6-d postinduction (Fig. 1, *C* and *D*). This reveals a marked change in the appearance of the nuclei in the induced cells, which are filled with condensed chromatin (Fig. 1, *C* and *D*, note dark nuclear staining in *D*).

A third series of control experiments were also carried out to measure the levels of three separate RNAs in uninduced MEL cells and at two, 4- and 6-d postinduction of terminal erythroid differentiation (Fig. 2). The levels of histone H4 and βmaj globin mRNAs and of U6 snRNA were determined at each stage using a nuclease S1 protection assay. This demonstrates that βmaj globin mRNA is not detected in the uninduced MEL cells, but is detected in cells at two days post induction and is present at high levels in cells at 4- and 6-d postinduction. In contrast, the histone H4 mRNA is detected readily in the uninduced MEL cells but the H4 mRNA level drops significantly during the course of differentiation. The U6 snRNA, which is a metabolically stable RNA species, is readily detected both before and after induction.

In summary, the combination of fluorescence and electron microscopy and RNA mapping data shown above confirm that the MEL cell line used in this study shows a similar response to DMSO induction as previously reported. Specifically, we have shown that, upon induction, the great majority of MEL cells in our cultures undergo the typical pattern of terminal erythroid differentiation, including activation of transcription of globin genes and a parallel transcriptional repression of other loci. The data further show that at the late stages of differentiation the MEL cell nuclei accumulate condensed chromatin, consistent with the previous observations that nascent transcription has ceased at this stage.

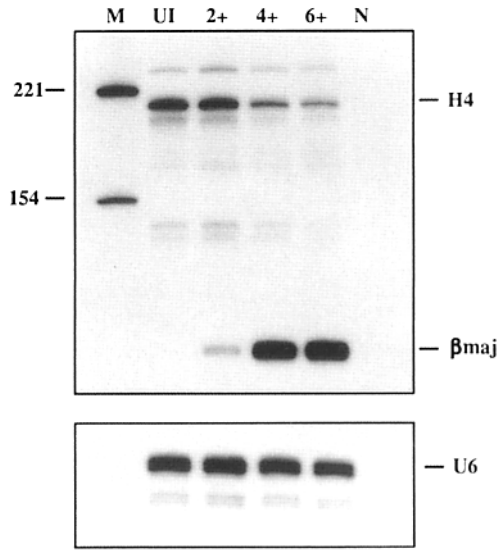


*Figure 2.* Effect of differentiation on RNA levels in MEL cells. Total RNA was extracted from uninduced (*UI*) MEL cells and from cells stimulated to undergo terminal erythroid differentiation in the presence of 2% DMSO for 2, 4, and 6 d. The levels of histone H4 mRNA (*H4*), βmajor-globin mRNA (*βmaj*) and U6 snRNA in each RNA preparation was analyzed by an S1-nuclease protection assay using end-labeled DNA probes as described in Materials and Methods. The sizes of the S1-nuclease protected fragments are 180 nucleotides (*nt*) for H4, 96 nt for βmaj and 87 nt for U6 RNAs, respectively. The samples analyzed for H4 and βmaj sequences contained 2 μg total RNA per reaction. Those analyzed for U6 snRNA contained 10 μg total RNA. An equivalent amount of *Escherichia coli* tRNA was used as a negative control (*N*) for each series of reactions. Size markers (*M*) are a HinfI digest of pBR322.

### *Localization of snRNPs in MEL Cells*

The distribution of splicing snRNPs in MEL cells was analyzed in uninduced cells and at 2- and 6-d postinduction of terminal erythroid differentiation (Fig. 3). The morphology of the MEL cells, as seen by phase contrast microscopy, is shown for the field of cells at each stage (Fig. 3, *A–C*). In the same cells, splicing snRNPs were detected using an anti-Sm mAb, which recognizes common snRNP proteins (Fig. 3, *D–F*). This shows that the snRNPs are concentrated in the nuclei of MEL cells, both before and after induction. In the uninduced cells, the snRNPs are widely distributed throughout the nucleoplasm, excluding nucleoli (Fig. 3 *D*). A broadly similar pattern is observed in cells at 2-d postinduction, though here a slightly more punctate staining is evident (Fig. 3 *E*). At 6-d postinduction, however, a significant change in the nuclear snRNP staining is seen in most cells (Fig. 3 *F*). At this late stage of differentiation, most of the snRNP is present in large clumps and much less widespread nucleoplasmic staining is visible.

We also examined the cells for the presence of coiled bodies at each of these stages (Fig. 3, *G–I*). The coiled bodies were detected using an anti-p80 coilin rabbit antiserum. Coiled bodies were seen in >95% of the cells, both before and after induction of terminal erythroid differentiation. Coiled bodies were detected in all the cells shown in Fig. 3 (*G–I*), but not all of them are visible in this figure because they occur in different focal planes in the separate cells in the field. A more detailed analysis of the relationship between the snRNP and coiled body staining patterns is presented in Fig. 5.

To obtain a higher resolution picture of nuclear snRNP distribution throughout MEL cell differentiation, the U1 and U2 snRNPs were analyzed individually (Fig. 4). They were detected by indirect immunofluorescence using U1 and U2-specific mAbs (Fig. 4, *E–L*). In addition, the U2 snRNA was independently localized by in situ hybridization using a complementary antisense 2′-O-alkyloligoribonucleotide (Fig. 4, *A–D*). This allows a comparison of the distribution pattern of the RNA and protein components of U2 snRNP. The distribution of each snRNP was examined in uninduced MEL cells and at 2-, 4-, and 6-d postinduction of terminal erythroid differentiation. Micrographs of representative, single cells are presented to reveal the nuclear staining pattern in more detail.

In the uninduced cells, both anti-U1 and -U2 snRNP probes show a widespread nucleoplasmic fluorescence, excluding nucleoli (Fig. 4, *A*, *E*, and *I*). This confirms the results obtained using the anti-Sm mAb (Fig. 3 *D*). For U2 snRNP, both the antibody and antisense probes reveal an identical labeling pattern, confirming that the assembled snRNP is being detected (Fig. 3, cf. *A* and *E*). Both probes also show that U2 snRNP is present in higher concentration in several discrete foci, as well as being widely distributed throughout the nucleoplasm (Fig. 4, *A* and *E*, *arrowheads*). However, U1 snRNP in comparison shows a predominantly

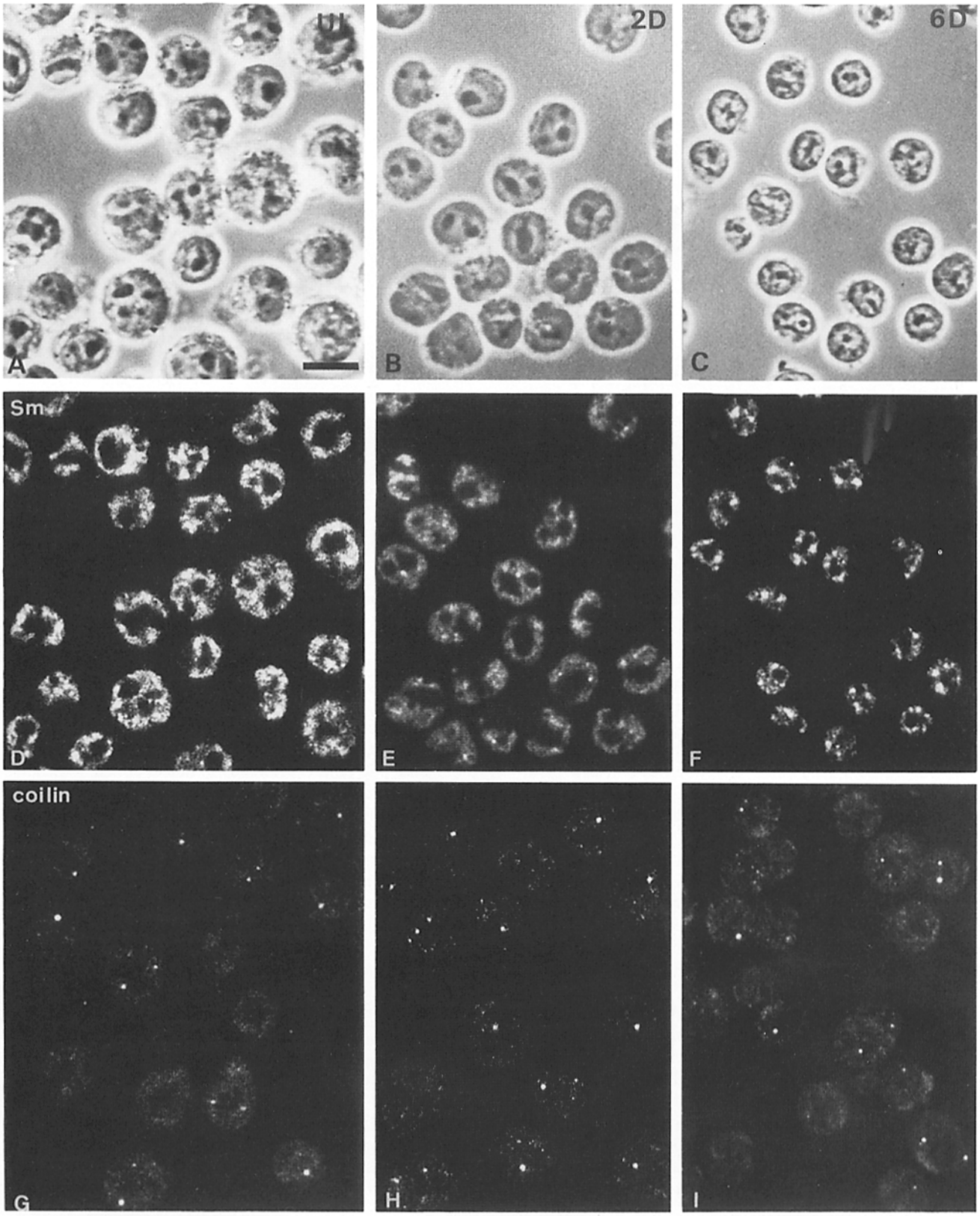


*Figure 3.* The effect of differentiation on the distribution of splicing snRNPs in MEL cells. Indirect immunofluorescence was performed with a monoclonal anti-Sm antibody (*D*, *E* and *F*) to detect splicing snRNPs in uninduced MEL cells (*D*) and at 2 (*E*) and 6 (*F*) d after terminal erythroid differentiation was induced by treatment with DMSO. *A*, *B*, and *C* represent the same fields of cells as seen by phase contrast microscopy. An anti-p80 coilin rabbit serum was also used to detect coiled bodies in uninduced MEL cells (*G*) and in cells at 2 (*H*) and 6 (*I*) d after induction to differentiate. Note that, due to the thickness of the nuclei, the number of coiled bodies observed in the single focal plane shown is not representative of the total number of coiled bodies per nucleus. Bar, 10 $\mu$m.

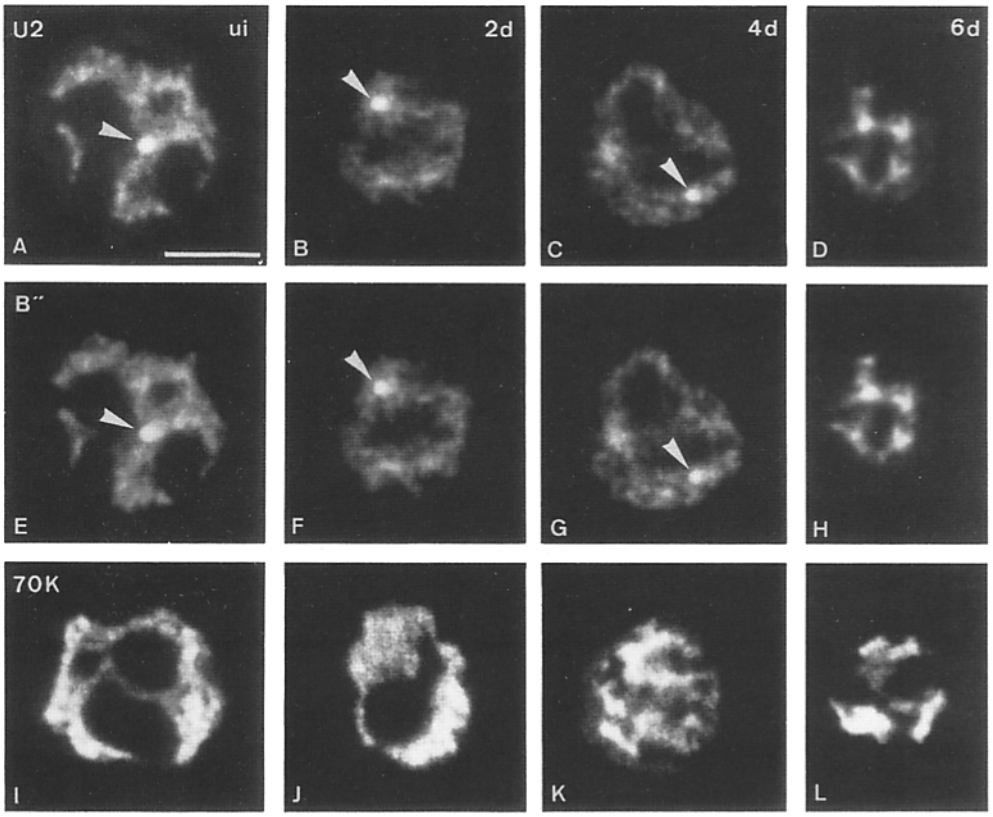


*Figure 4.* Localization of U1 and U2 snRNPs in MEL cells. Both uninduced MEL cells (*A*, *E*, and *I*), and cells at 2 (*B*, *F*, and *J*), 4 (*C*, *G*, and *K*), and 6 (*D*, *H*, and *L*) days after terminal erythroid differentiation was induced by treatment with DMSO, were analyzed by a combination of in situ hybridization and indirect immunofluorescence. The U2 snRNP was studied in parallel with a U2 snRNA-specific antisense probe (*A–D*) and a mAb against the U2 snRNP-specific B″ protein (*E–H*). The distribution of U1 snRNP was analyzed using a mAb directed against the U1 snRNP-specific 70-kD protein (*I–L*). Bar, 5 μm.

widespread nucleoplasmic distribution without the striking concentration in foci (Fig. 4, *I*). This picture of the nuclear distributions of splicing snRNPs in MEL cells is in close agreement with previous double-labeling studies on the U1 and U2 snRNPs in HeLa cells (Carmo-Fonseca et al., 1991*a*,*b*, 1992; Matera and Ward, 1993) and with in situ hybridization analysis of U1 and U2 snRNAs at the EM level (Visa et al., 1993).

After induction of terminal erythroid differentiation, changes are evident in the pattern of nuclear staining for both U1 and U2 snRNPs, particularly at the late stage (Fig. 4, *B–D*, *F–H*, and *J–L*). At 2- and 4-d postinduction, both the antisense and antibody probes still show some of the U2 snRNP concentrated in discrete foci (Fig. 4, *B*, *C*, *F*, and *G*, *arrowheads*). However, at later stages postinduction, particularly at the 6-d stage, both U1 and U2 snRNPs show a more prominently clumped pattern and appear to stain less of the nucleoplasm as compared with uninduced cells (Fig. 4, compare *A*, *E*, and *I*, with *D*, *H*, and *L*). The antibody and antisense probes for U2 snRNP give identical staining patterns at each time point postinduction, confirming that a genuine rearrangement in the localization of U2 snRNP is taking place during differentiation, rather than a dissociation and rearrangement of U2 snRNP protein independent of U2 snRNA (Fig. 4, cf. *B* and *F*, *C* and *G*, and *D* and *H*).

### *Association of Splicing snRNPs with Coiled Bodies during Differentiation*

The bright foci of concentrated snRNP staining in MEL cells have a similar appearance to the snRNP foci detected in other cell lines, which were shown to be coiled bodies (Raska et al., 1991; Andrade et al., 1991; Carmo-Fonseca et al., 1992; Huang and Spector, 1992). To determine whether the snRNP foci in MEL cells also correspond to coiled bodies, double-labeling experiments were performed using anti-p80 coilin antibodies to detect coiled bodies and an anti-Sm mAb to detect splicing snRNPs (Fig. 5). The individual staining patterns of the Sm and anti-p80 coilin antibodies in uninduced MEL cells were recorded in the confocal laser scanning fluorescence microscope (Fig. 5, *A* and *B*). An overlay of the separate images (Fig. 5 *C*), demonstrates that the bright snRNP foci correspond to the coiled bodies labeled by the anti-p80 coilin antibody (Fig. 5, cf. *A–C*, *arrowheads*; note yellow foci in *C* due to the superimposition of red and green staining).

A similar double-labeling analysis was carried out to assess the association of splicing snRNPs with coiled bodies in MEL cells at two, four and 6-d postinduction of terminal erythroid differentiation (Fig. 5, *D–F*). In this case the figure shows only the overlays from the double labeling and not the separate anti-Sm and anti-p80 coilin staining at each stage. At 2 d (Fig. 5 *D*) and 4 d (Fig. 5 *E*) postinduction yellow foci are detected. This demonstrates that splicing snRNPs continue to associate with coiled bodies after differentiation is induced (cf. Fig. 3, *G–I*). However, at 6-d postinduction (Fig. 5 *F*), the foci are red and the snRNP staining is concentrated in large clumps that are not stained by the anti-p80 coilin antibody. This indicates that snRNPs no longer associate with coiled bodies at the late stages of terminal erythroid differentiation. This corresponds to the stage when transcription has ceased and chromatin is condensing (Sherton and Kabat, 1976; Orkin and Swerdlow, 1977; Patel and Lod-

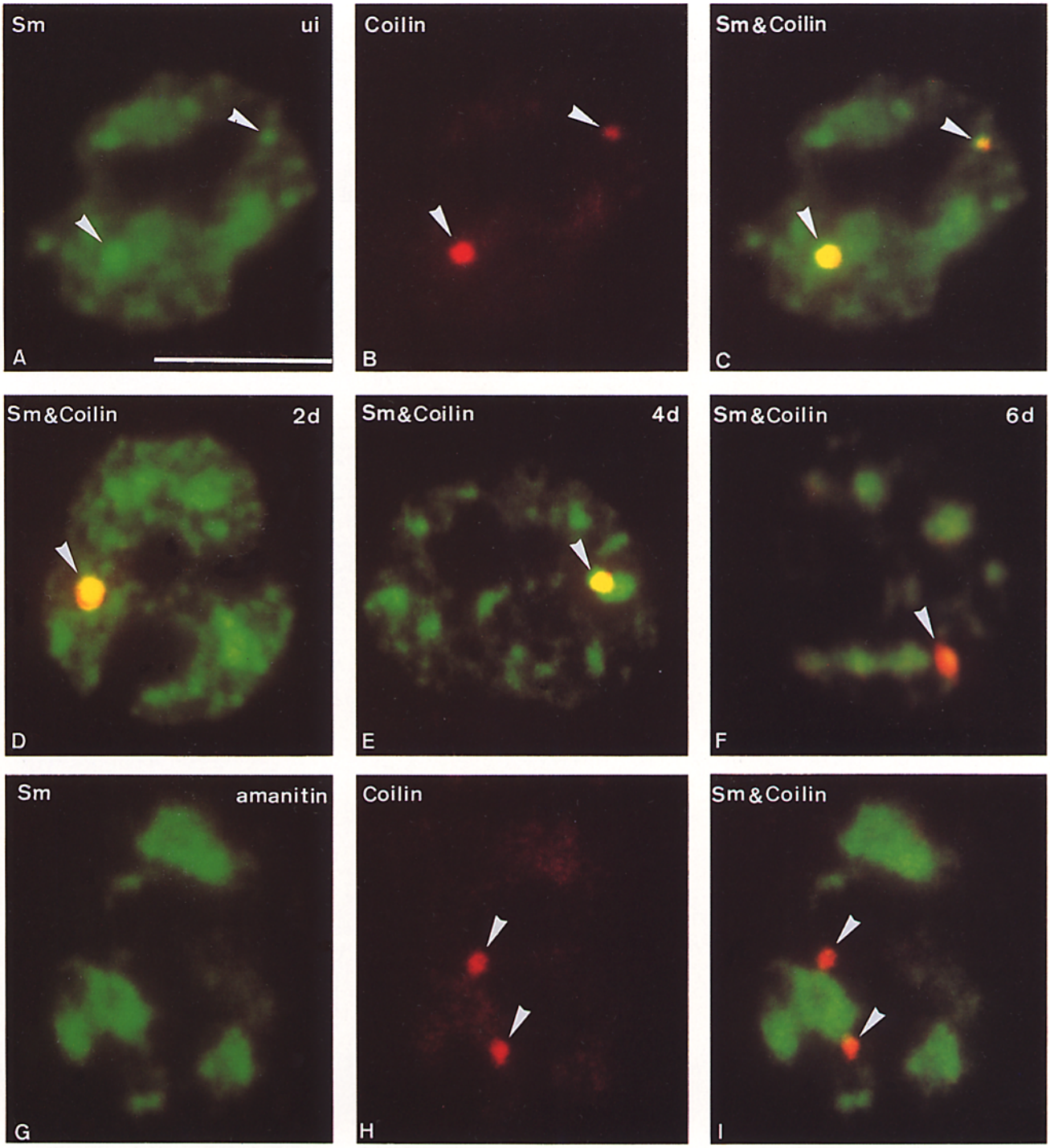


*Figure 5.* snRNP association with coiled bodies in MEL cells. Double-labeling experiments were performed to assess whether splicing snRNPs are present in coiled bodies in MEL cells. snRNPs were detected using a monoclonal anti-Sm antibody (*green*) and coiled bodies using an anti-p80 coilin antibody (*red*) in uninduced MEL cells (*A–C*), and in cells at 2 (*D*), 4 (*E*), and 6 (*F*) d after terminal erythroid differentiation was induced by treatment with DMSO. The association of snRNPs with coiled bodies was also examined in uninduced cells after transcription was blocked by treatment with α-amanitin (*G–I*). Overlays of the anti-Sm and anti-coilin staining patterns are shown in *C*, *D*, *E*, *F*, and *I*. In uninduced cells, and at *2* and *4* d postinduction, both antibodies colocalize in coiled bodies (*yellow staining* indicated by *arrowheads* in *C*, *D*, and *E*). At 6-d postinduction, and in uninduced cells treated with α-amanitin, the Sm antigens are not detected in coiled bodies (*red* staining indicated by *arrowheads* in *F* and *I*). Bar, 5 μm.

ish, 1984; see also the electron micrographs in Fig. 1 of this study).

The change in snRNP distribution seen in MEL cells at the late stages of differentiation, including the loss of snRNPs from coiled bodies, is similar to the change in snRNP distribution in HeLa cells when they are treated with inhibitors of RNA polymerase II (Carmo-Fonseca et al., 1992). Therefore, to test whether the reduced level of transcription at the late stages of differentiation may be responsible, at least in part, for the change in snRNP distribution, uninduced MEL cells were analyzed after treatment with the RNA polymerase II inhibitor $\alpha$-amanitin (Fig. 5, *G–I*). Staining with anti-Sm antibodies (Fig. 5 *G*), shows that treatment of MEL cells with $\alpha$-amanitin also results in a redistribution of snRNPs, which become concentrated in large clumps and are no longer dispersed throughout the nucleoplasm (Fig. 5, cf. *A* and *G*). Double-labeling with anti-p80 coilin antibodies shows that coiled bodies can still be detected (Fig. 5 *H*).

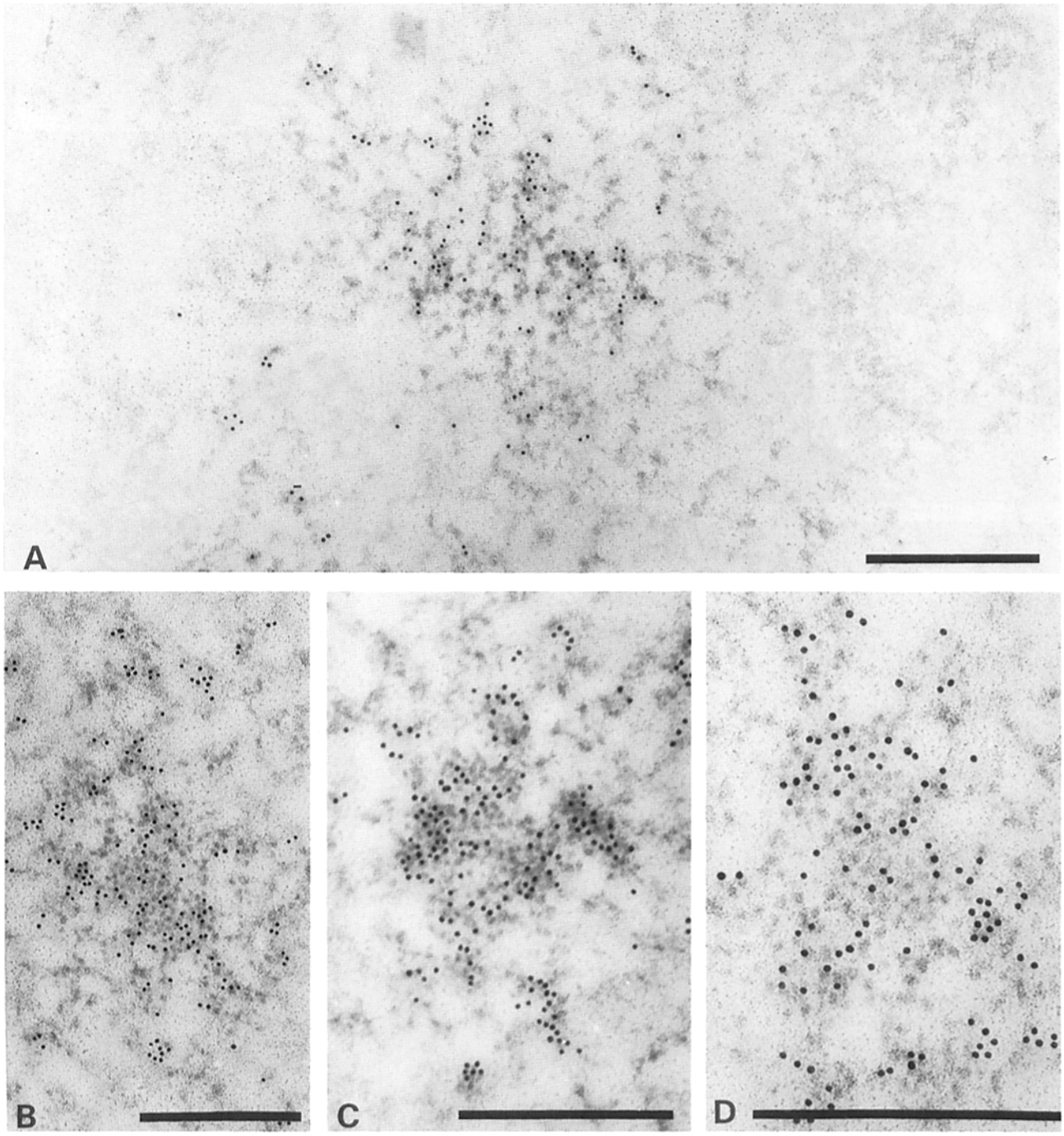


*Figure 6.* The mAb 3C5 specifically labels interchromatin granule clusters in MEL cells. Ultra-thin sections of uninduced MEL cells were immunogold labeled with the mAb 3C5 and stained by the EDTA regressive method of Bernhard (Bernhard, 1969). EM shows that the gold label is specifically concentrated over clusters of interchromatin granules (*A–D*). Bar, 0.5 $\mu$m.

An overlay of images Fig. 5 (*G* and *H*) shows that these coiled bodies are not stained by the anti-Sm antibodies, indicating that treatment with α-amanitin has resulted in an exodus of snRNPs from coiled bodies (Fig. 5 *I*, *arrowheads*; note red coiled bodies). However, the coiled bodies are observed intimately juxtaposed beside snRNP clusters. This organization was frequently observed in MEL cells and has also been seen in HeLa cells following treatment with α-amanitin (Carmo-Fonseca et al., 1992). This suggests that snRNPs move from the coiled bodies into the large clusters when transcription is halted. As the redistribution of snRNPs observed in MEL cells following α-amanitin treatment is similar to that observed at late time points after induction to differentiate, the reduced levels of transcription in both cases may be largely responsible for the changes in snRNP localization.

### *SnRNPs Concentrate in Clusters of Interchromatin Granules at Late Stages of Differentiation*

In interphase cells, the speckled, or punctate, nuclear structures containing splicing snRNPs arise from the association of snRNPs with clusters of interchromatin granules as well as with coiled bodies (reviewed by Lamond and Carmo-Fonseca, 1993*b*). Since the large clumps of snRNP observed at late stages of differentiation are not coiled bodies, we therefore examined whether they might correspond instead to clusters of interchromatin granules. To test this we used mAb 3C5, a mAb that was previously shown to predominantly stain interchromatin granules in mammalian cells (Turner and Franchi, 1987). First, we confirmed by immunoelectron microscopy that mAb 3C5 also stains interchromatin granules in MEL cells (Fig. 6). The interchromatin granules were revealed by the EDTA regressive method of Bernhard (Bernhard, 1969). Analysis of ultra-thin sections immunogold labeled with mAb 3C5 shows that the staining is highly specific for interchromatin granules.

Having confirmed its specificity in MEL cells, mAb 3C5 was then used to double-label MEL cells for snRNPs and interchromatin granules (Fig. 7). Splicing snRNPs were detected using an anti-Sm mAb (Fig. 7, *A* and *B*). Double labeling with two mouse mAbs was possible in this case since mAb 3C5 is an IgM and the anti-Sm mAb (Y12) is an IgG. The anti-Sm staining again shows the striking rearrangement of splicing snRNPs from a widespread nucleoplasmic distribution in uninduced MEL cells to a restricted pattern of clumps at 6-d postinduction of terminal erythroid differentiation (Fig. 7, *A* and *B*, cf. Figs. *3–5*). In contrast, with mAb 3C5 a similar punctate pattern of nucleoplasmic staining is observed both in uninduced cells and at 6-d postinduction, although the structures stained can appear larger in cells at late stages postinduction (Fig. 7, *C* and *D*). However, at 6-d postinduction the anti-Sm and 3C5 mAbs now colocalize in the same punctate structures (Fig. 7, cf. *B* and *D*). This contrasts with the uninduced cells, where the snRNP staining is widespread in the nucleoplasm and clearly not confined to the clusters of interchromatin granules, i.e., the punctate structures stained by mAb 3C5 (Fig. 7, cf. *A* and *C*).

In summary, the data show that the large clumps of snRNP staining that accumulate in the nuclei of MEL cells at late stages of terminal erythroid differentiation correspond to clusters of interchromatin granules. Since only a subset of the splicing snRNPs are associated with interchromatin granule clusters in uninduced cells, this indicates that snRNPs move out of other nuclear structures (including coiled bodies) and into clusters of interchromatin granules at late stages postinduction.

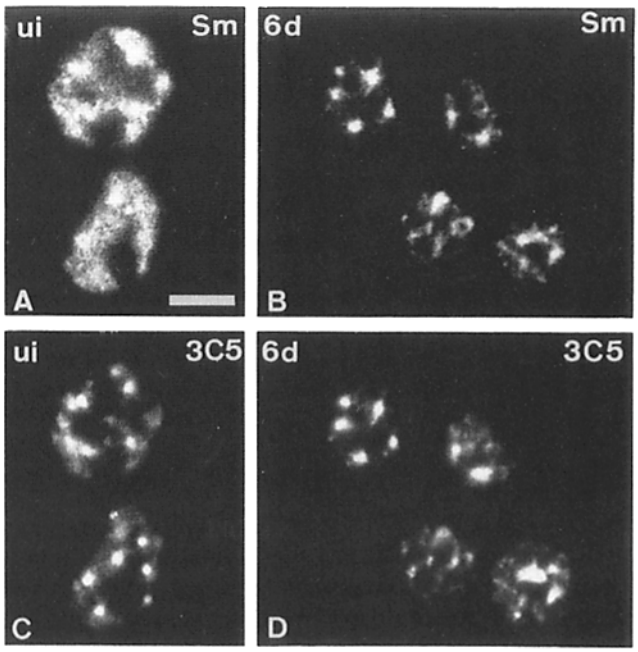


*Figure 7.* snRNPs accumulate in large clusters of interchromatin granules at late stages of MEL cell differentiation. Double-labeling experiments were performed to determine the localization of splicing snRNPs and of interchromatin granule clusters in uninduced MEL cells (*UI*) and at 6 d after terminal erythroid differentiation was induced by treatment with DMSO. The Y12 anti-Sm mAb (IgG) was used to detect splicing snRNPs (*A* and *B*) and the 3C5 monoclonal antibody (IgM) was used to detect interchromatin granule clusters (*C* and *D*). In uninduced cells the snRNPs show a typical widespread nucleoplasmic distribution (cf. Figs. 3–5) while the interchromatin granules are seen as a more restricted pattern of punctate nucleoplasmic staining (compare *A* and *C*). At 6-d postinduction both antibodies co-localize in several large nucleoplasmic clusters (compare *B* and *D*). Bar, 5 μm.

### *Localization of Splicing Factor SC-35 during MEL Cell Differentiation*

Recent studies have established that a mAb, termed SC-35, recognizes a non-snRNP protein required for pre-mRNA splicing in mammalian splicing extracts (Fu and Maniatis, 1990). This antibody gives a punctate staining pattern in HeLa cells and EM analysis showed that it labels both clusters of interchromatin granules and perichromatin fibrils (Spector et al., 1991). It does not, however, label coiled bodies (reviewed by Lamond and Carmo-Fonseca, 1993*a*). The SC-35 mAb also stains the nuclei of uninduced MEL cells in a punctate pattern (Fig. 8 *A*). A similar punctate staining pattern is seen with mAb SC-35 at 6-d postinduction of terminal erythroid differentiation (Fig. 8 *B*). The major difference between the SC-35 staining patterns before and after induction is that fewer but larger structures are often labeled at late stages postinduction. The mAb SC-35 labels the same

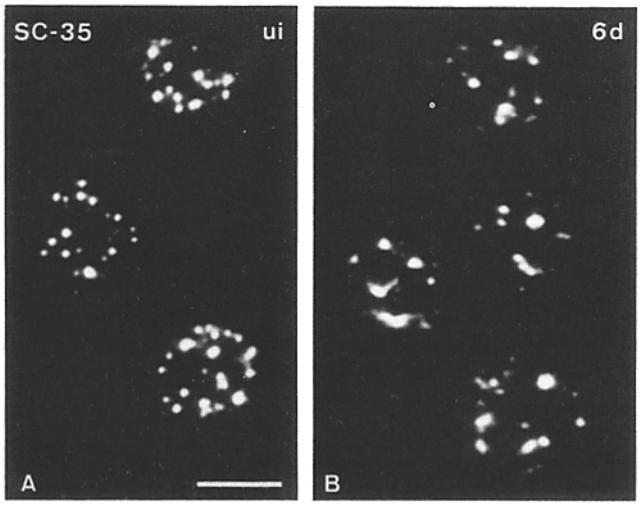


*Figure 8.* Distribution of the splicing factor SC-35 in MEL cells. The mAb SC-35 gives a punctate nucleoplasmic staining pattern in both uninduced MEL cells (*A*) and at 6 d after terminal erythroid differentiation was induced by DMSO treatment (*B*). In *B* the punctate structures appear larger and fewer in number than in the transcriptionally active, uninduced cells shown in *A*. Bar, 5 μm.

structures in MEL cells as does mAb 3C5, both before and after induction (data not shown, cf. Figs. 7 and 8). This is consistent with previous observations comparing the labeling patterns of mAbs SC-35 and 3C5 in HeLa cells (Carmo-Fonseca et al., 1992). The data, therefore, indicate that mAb SC-35 strongly stains clusters of interchromatin granules in MEL cells, both before and after induction of erythroid differentiation. This is in agreement with the earlier data on SC-35 localization arising from EM studies (Spector et al., 1991).

### *Splicing snRNPs Are Segregated into Membrane Associated Domains prior to Enucleation*

The final stage of erythroid differentiation undergone by MEL cells is marked by a global condensation of chromatin, followed by enucleation (Volloch and Housman, 1982; Patel and Lodish, 1987). We therefore analyzed cells at this extreme terminal stage, using a combination of fluorescence and electron microscopy, to determine what happened to snRNPs and other nuclear antigens (Figs. 9 and 10).

The appearance of MEL cells is illustrated at the extreme terminal stage postinduction, as shown by phase contrast microscopy (Fig. 9 *A*). For analysis by confocal fluorescence microscopy, splicing snRNPs were labeled using three separate probes; an anti-Sm mAb (Fig. 9 *B*), a U2 snRNP-specific mAb (Fig. 9 *E*) and an antisense probe specific for U2 snRNA (Fig. 9 *D*). All three probes reveal that the splicing snRNPs are located predominantly in a large aggregate at the periphery of the condensed chromatin (Fig. 9, *B*, *D*, and *E*, *arrows*). In addition, a lower level of staining is often observed around the nuclear periphery (Fig. 9, *B*, *D*, and *E*, *arrowheads*). Double labeling the cells stained with the anti-Sm monoclonal (Fig. 9 *B*), with mAb 3C5 (Fig. 9 *C*), shows that the same large structure at the nuclear periphery is labeled by both antibodies. This suggests that it contains a super cluster of interchromatin granules. In contrast, cells labeled with anti-p80 coilin antibodies show more widespread staining around the nuclear periphery without the striking concentration in a single structure (Fig. 9 *F*). At this stage there is no longer any sign of discrete coiled bodies.

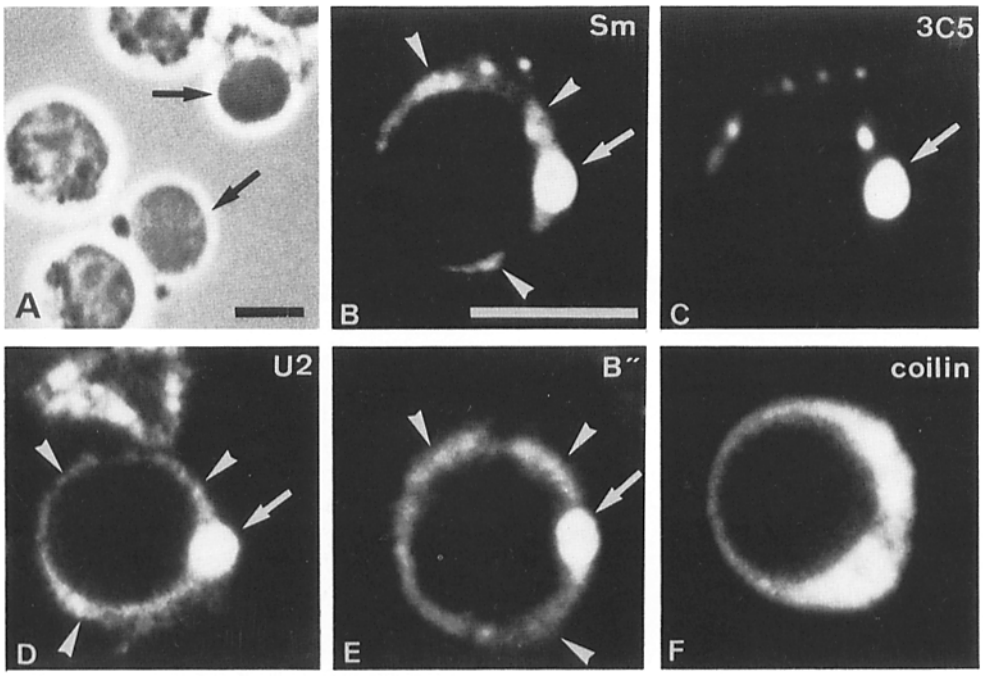


*Figure 9.* The distribution of snRNPs at terminal enucleation of MEL cells. The terminal stage of MEL cell differentiation is marked by a global condensation of chromatin, as seen by phase contrast in *A* (see also Fig. 1 *D*). Splicing snRNPs were localized at this terminal stage of erythroid differentiation using three separate probes; an Sm-specific mAb (*B*), a U2 snRNA-specific antisense probe (*D*), and a mAb specific for the U2 snRNP protein B″ (*E*). All three probes show that the snRNPs are predominantly localized within a single large aggregate at the periphery of the condensed chromatin (*B*, *D*, and *E*, *arrows*) with a lesser amount of staining around the rest of the periphery (*B*, *D*, and *E*, *arrowheads*). The large snRNP aggregate is also stained by the mAb 3C5 (*C*), as shown by double labeling (compare *B* and *C*, *arrows*). In contrast, the coiled body antigen, p80 coilin, is not confined to the snRNP aggregate but rather shows more widespread staining around the periphery of the condensed chromatin (*F*). Bar, 5 μm.

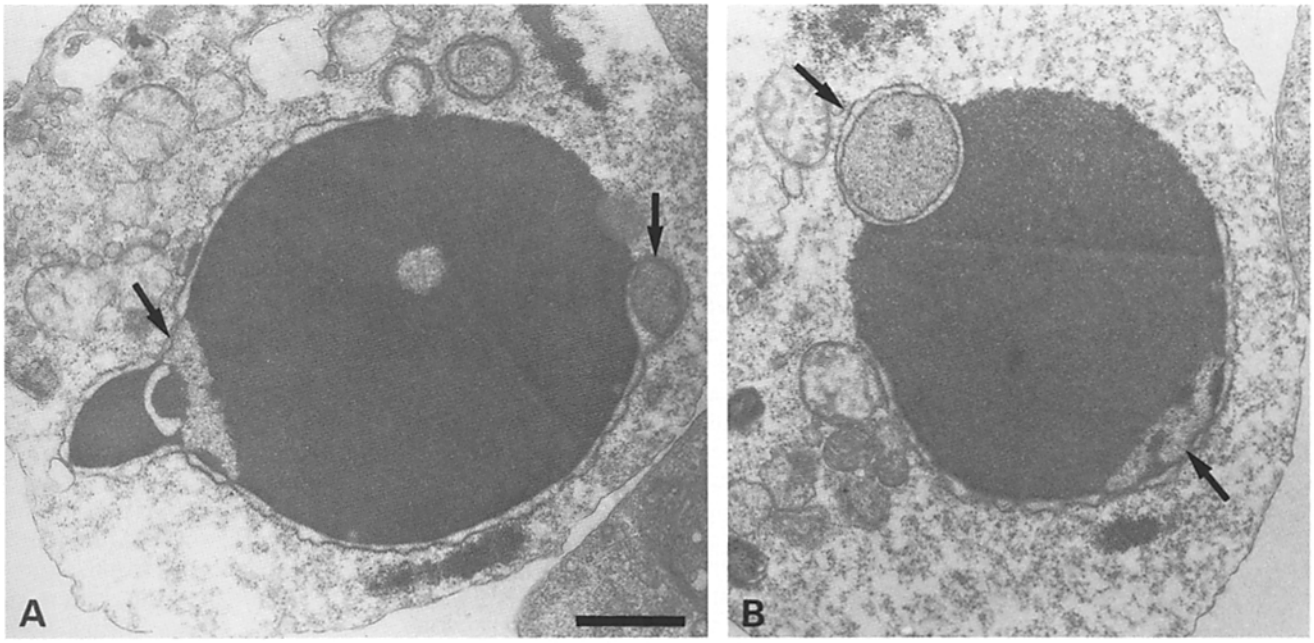


*Figure 10.* EM of differentiating MEL cells at the terminal stage just before enucleation. Ultrathin sections of terminally differentiated MEL cells were stained by conventional methods and examined in the electron microscope. The nuclei are full of condensed chromatin and show regions, mainly at the nuclear periphery, where nonchromatin components appear to have been segregated (*arrows*). Note the partial loss of membrane from the nuclear periphery and the presence of membrane enclosed domains from which condensed chromatin is excluded. Bar, 1 $\mu$m.

The structure of MEL cell nuclei at the extreme terminal stage postinduction was also examined in the electron microscope (Fig. 10). In all cases the nuclei are densely packed with condensed chromatin. The prominent snRNP-containing structures at the periphery of the condensed chromatin are clearly visible. Surprisingly, these structures are surrounded by a membrane. This is most likely derived from the nuclear envelope, which is largely absent from the nuclear periphery in the vicinity of the snRNP structures. Therefore, after chromatin condensation, it appears that the splicing snRNPs, (and probably other nuclear antigens), migrate to the nuclear periphery, associate with the nuclear envelope and form a discrete, membrane enclosed domain. We propose the name "SCIM" domain (i.e., SnRNP Clusters Inside a Membrane), to describe this novel structure.

## Discussion

In this study we have analyzed the distribution of splicing snRNPs in the nuclei of MEL cells, both before and after induction of terminal erythroid differentiation in vitro. The data show that splicing snRNPs are not dramatically reorganized within the first two days after induction to differentiate, during which time a major switch in the pattern of gene expression takes place, including the repression of previously active loci and the activation of erythroid-specific transcription. In both uninduced MEL cells, and at early stages following induction, splicing snRNPs are widely distributed throughout the nucleoplasm and show enhanced concentrations in bright foci that correspond to coiled bodies. However, four to six days after MEL cells are induced to differentiate, this widespread nucleoplasmic snRNP staining markedly changes. At the late stage of differentiation, when transcription has ceased, splicing snRNPs no longer associate with coiled bodies and concentrate instead in large aggregates that correspond to clusters of interchromatin granules.

A major conclusion from the present study is that splicing snRNPs can be induced to change their distribution following a differentiation process that alters the pattern and level of gene expression. However, the major changes in snRNP localization are observed only after a global decrease in the level of transcription at late stages of differentiation and not at the earlier stages postinduction when a profound alteration takes place in the pattern of genes being transcribed. The observation that the number of coiled bodies per cell decreases from an average of three to four in uninduced cells, to an average of less than 2- at 6-d postinduction (data not shown), also suggests that the association of splicing snRNPs with this structure is more dependent on the total level, rather than the qualitative pattern, of ongoing transcription. The absence of a clear change in snRNP distribution immediately after induction was somewhat unexpected and has interesting implications for the spatial organization of active genes in the nucleus of MEL cells and their relationship with splicing factors. The data may be explained in three alternative models of nuclear organization: (*a*) in MEL cells there is no special relationship between gene organization and accessibility to splicing snRNPs, i.e., snRNPs can readily access any newly transcribed gene wherever it is located in the nucleoplasm; (*b*) a major reorganization of chromatin occurs following the induction of MEL cells to differentiate, to bring previously inactive loci (such as globins) to specific subnuclear locations where snRNPs can interact with them; or (*c*) in uninduced MEL cells, the loci which are only transcribed follow-

ing differentiation are already assembled into "active" sites together with genes that are already being transcribed and processed. Studies are now underway to try and distinguish between these alternative possibilities by comparing the spatial organization of specific genes, relative to splicing snRNPs, before and after MEL cell differentiation.

The snRNP staining pattern in MEL cells markedly rearranges when transcription halts, i.e., at late times after induction of differentiation or in undifferentiated cells treated with the transcription inhibitor α-amanitin. While changes in snRNP staining have been observed previously in cells treated with drugs, or after heat shock (Carmo-Fonseca et al., 1991*a*,*b*, 1992; Spector et al., 1991), we provide here a clear demonstration that snRNP localization is also affected by the physiological changes accompanying differentiation. MEL cell differentiation results in a change in the steady state level of snRNPs associated with distinct subnuclear compartments. For example, the association of snRNPs with coiled bodies is a prominent feature in uninduced MEL cells, but is lost at late stages of differentiation when transcription has ceased. These data are consistent with the view that the interaction of snRNPs with coiled bodies is of functional significance for some aspect of their role in the pathway of pre-mRNA processing or transport (reviewed by Lamond and Carmo-Fonseca, 1993*a*). This does not imply that coiled bodies must be direct sites of pre-mRNA splicing in MEL cells. The coiled bodies may instead play other roles required for the participation of snRNPs in the splicing pathway, e.g., snRNP or spliceosome assembly or disassembly, intron degradation or some aspect of intranuclear snRNP transport connected with gene expression. However, the data argue against coiled bodies being storage sites for inactive snRNPs, since snRNPs no longer associate with coiled bodies at the late stage of differentiation when the proportion of inactive snRNPs in the nucleus must increase.

Splicing snRNPs concentrate specifically in large clusters of interchromatin granules at late stages of differentiation, or when transcription is inhibited by drug treatment. A subset of snRNPs also associate with interchromatin granule clusters when transcription and splicing are taking place and this contributes to the punctate staining pattern frequently observed with anti-snRNP antibodies. The present data indicate that snRNPs can move between coiled bodies and interchromatin granule clusters in MEL cell nuclei. It is possible that snRNPs engaged in the various steps associated with pre-mRNA maturation are normally in flux between separate subnuclear locations, including coiled bodies and interchromatin granules (reviewed by Lamond and Carmo-Fonseca, 1993*a*). Such a dynamic movement of snRNPs could take place as nascent transcripts are recognized, spliceosomes assembled, mRNA formed and exported to the cytoplasm, and introns degraded and the snRNPs recycled for further rounds of splicing activity. Separate (though possibly overlapping) activities may take place in coiled bodies and interchromatin granules, respectively. Identifying which activities occur in each compartment is an important goal for future studies. However, it should again be noted that neither structure need be the actual site of splicing. Since there is a very strong widespread nucleoplasmic staining in MEL cells, splicing may take place on nascent transcripts in the interchromatin space outside of either interchromatin granules or coiled bodies. This would be consistent with EM data showing that splicing can take place on nascent transcripts of *Drosophila* pre-mRNA (Beyer and Osheim, 1988).

Since after MEL cells are induced to differentiate individual genes are switched on and expressed at high levels, while the overall number of different transcripts being synthesized decreases, this provides a useful model system for studying the localization of specific pre-mRNAs at different stages of the processing pathway. MEL clones which can be induced to express wild type and mutant β-globin transcripts at high levels (Collis et al., 1990; Antoniou and Grosveld, 1990), may help to identify subnuclear sites at which splicing and transport events take place. In this regard, the present analysis of snRNP localization represents an important preliminary study which should aid the interpretation of future analyses of mRNA localization.

At the extreme terminal stage of erythroid differentiation we have observed that splicing snRNPs, the coiled body protein p80 coilin and antigens stained by mAb 3C5, are all excluded from the condensed, transcriptionally inactive chromatin before enucleation. The snRNPs (but not coilin) predominantly cluster in a membrane bound structure at the nuclear periphery. We have termed this novel structure a "SCIM" domain. Splicing snRNPs and other nuclear antigens are also displaced from chromosomes when they condense during mitosis (Verheijen et al., 1986; Leser et al., 1989). However, during mitosis snRNPs do not form the large membrane bound domains we observe before enucleation. Rather, they are mostly widely distributed throughout the mitotic cytoplasm. This may reflect the fact that, unlike the events preceding enucleation, chromatin condensation, and other changes in nuclear structure occurring during mitosis are reversible phenomena.

It is not clear whether the snRNPs, and any other nuclear antigens in the SCIM domain, are actually expelled from the MEL cells during enucleation. Although we do not detect snRNP antigens in mature erythrocytes (unpublished observations), we cannot exclude that the SCIM domain is retained and subsequently degraded within the erythrocyte after the condensed chromatin is expelled. Given the significant degree of breakdown of the nuclear envelope we have observed before enucleation (see Fig. 10), it is apparent that loss of the "nucleus" from MEL cells does not correspond to the ejection of an entirely membrane enclosed organelle comparable to the interphase nucleus. Our data raise the possibility that "enucleation" may actually correspond to the ejection from the cell of an aggregate of condensed chromatin, from which many other components of the interphase nucleus have already been segregated. In this case the formation of the SCIM domain may play an important role in the mechanism that results in mammalian erythrocytes losing their nuclei. It will now be interesting to determine whether an analogous SCIM domain is formed at the terminal stage of avian erythroid differentiation, since nuclei are not lost from avian erythrocytes.

Whatever role it may play in the enucleation process, formation of the SCIM domain represents a striking example of a molecular sorting process involving splicing snRNPs. It also raises a number of interesting questions regarding the mechanism of the sorting process itself and, importantly, the mechanism whereby a similar effect is prevented from occurring before the extreme terminal stage of erythroid differenti-

ation. These observations underline the inherent complexity of the mammalian nucleus and illustrate the potential for regulatory mechanisms that determine the association and structural organization of nuclear components.

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