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Associations between distinct pre-mRNA splicing components and the cell nucleus

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SC-35 is a non-snRNP spliceosome component that is specifically recognized by the anti-spliceosome monoclonal antibody αSC-35. In this paper we provide direct evidence that SC-35 is an essential splicing factor and we examine the immunolocalization of SC-35 by confocal laser scanning microscopy and by electron microscopy. We have found that the speckled staining pattern observed by fluorescence microscopy corresponds to structures previously designated as interchromatin granules and perichromatin fibrils. Although snRNP antigens are also concentrated in these nuclear regions, we show that the two types of spliceosome components are localized through different molecular interactions: The distribution of SC-35 was not affected by treatment with DNase I or RNase A, or when the cells were heat shocked. In contrast, snRNP antigens become diffusely distributed after RNase A digestion or heat shock. Examination of cells at different stages of mitosis revealed that the SC-35 speckled staining pattern is lost during prophase and speckles containing SC-35 begin to reform in the cytoplasm of anaphase cells. In contrast, snRNP antigens do not associate with speckled regions until late in telophase. These studies reveal a dynamic pattern of assembly and disassembly of the splicing factor SC-35 into discrete nuclear structures that colocalize with interchromatin granules and perichromatin fibrils. These subnuclear regions may therefore be nuclear organelles involved in the assembly of spliceosomes, or splicing itself.
Key words: nuclear structure/RNA processing/snRNP particles/spliceosome

Introduction

Nuclear pre-mRNA splicing takes place in spliceosomes, which are composed of small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4/U6, U5 (for a review see Krainer and Maniatis, 1988; Steitz *et al.*, 1988; Bindereif and Green, 1990) and a large number of associated non-snRNP proteins (Reed, 1990). A number of non-snRNP splicing activities have been identified by *in vitro* complementation experiments (Krainer and Maniatis, 1988; Bindereif and Green, 1990 for reviews), but only three have been purified to homogeneity. One of these factors, designated SFII (Krainer and Maniatis, 1985; Krainer *et al.*, 1990) or ASF (Ge and Manley, 1990) is required for spliceosome assembly, and it plays a role in 5′ splice site selection. The second factor, U2AF, facilitates U2 snRNP binding to the 3′ splice site (Ruskin *et al.*, 1988; Zamore and Green, 1989, 1991). The third factor, an 88 kDa protein, was identified by a monoclonal antibody directed against large nuclear ribonucleoprotein particles (Ast *et al.*, 1991). A spliceosomal protein, designated SC-35, was identified using a monoclonal antibody directed against partially purified spliceosomes (Fu and Maniatis, 1990). Extracts depleted of this protein fail to carry out the first step of the splicing reaction, and do not form spliceosomes (Fu and Maniatis, 1990). However, these depleted extracts can be complemented with extracts containing SC-35 but not by those which do not contain this antigen (Fu and Maniatis, 1990).

Although significant advances have been made in understanding basic splicing mechanisms and progress is being made in identifying functional components of the spliceosome, relatively little is known about the organization of the splicing apparatus in the nucleus. Individual splicing factors could be soluble components of the nucleoplasm that are assembled on nascent pre-mRNA. Alternatively, spliceosome assembly and or splicing itself could be compartmentalized in the nucleus. The possibility that splicing components are compartmentalized was first suggested by immunofluorescent staining experiments using anti-snRNP or anti-snRNA antibodies (Spector *et al.*, 1983; Spector, 1984; Reuter *et al.*, 1984; Nyman *et al.*, 1986; Verheijen *et al.*, 1986). These studies revealed diffuse nuclear staining superimposed on 20–50 highly concentrated regions which appeared as a 'speckled' staining pattern. Subsequently, three-dimensional (3-D) reconstruction techniques were used to show that snRNPs are distributed within a reticular network that extends between the nucleolar surface and the nuclear lamina-envelope (Spector, 1990). This network could correspond to sites of snRNP assembly or storage.

Recently, immunofluorescent staining experiments with the αSC-35 antibody revealed that SC-35 colocalizes with snRNPs within the speckled nuclear domains (Fu and Maniatis, 1990). This observation provided the first evidence that the speckled regions contain non-snRNP spliceosome components. The speckled regions are therefore more than sites of snRNP assembly or storage. They may correspond to sites of spliceosome assembly or splicing itself.

In this paper we provide definitive evidence that SC-35 is a splicing factor, and we compare the nuclear localization of snRNP antigens with SC-35 by immunofluorescence and immunoelectron microscopy. We find that these spliceosome components colocalize to nuclear regions previously designated interchromatin granules and perichromatin fibrils (for a review, see Fakan and Puvion, 1980; Puvion and Moyne, 1981; Raska *et al.*, 1990). However, these factors appear to be associated with the nucleus by different molecular interactions, since they are affected differently by RNase digestion and heat shock. During mitosis, a time in

the cell cycle when the nuclear envelope breaks down and transcription is shut off, the nuclear regions involved in RNA processing break up and splicing factors are redistributed throughout the cytoplasm. The reformation of the nuclear domains in which splicing factors are concentrated begins late in anaphase. At this time, SC-35 begins to form a speckled pattern in the cytoplasm, while snRNP antigens are more uniformly distributed throughout the cytoplasm. By late telophase, when the nuclear envelope has reformed, both SC-35 and snRNP antigens are once again colocalized within a speckled nuclear network.

These observations demonstrate clear differences in the nuclear associations of snRNP antigens and SC-35 and their temporal patterns of assembly during mitosis. The results presented here, and recent studies of the nuclear distribution of SC-35 and snRNPs in amphibian germinal vesicles (Wu *et al.*, 1991), add further support to the hypothesis that the speckled nuclear domains are sites of spliceosome assembly and/or splicing.

Results

Immunoaffinity-purified SC-35 is an active splicing factor

We previously showed that the αSC-35 mAb efficiently immunoprecipitates purified spliceosomes, and it inhibits splicing *in vitro* (Fu and Maniatis, 1990). In addition, SC-35-depleted nuclear extracts are incapable of splicing exogenously added pre-mRNA, but can be complemented by extracts containing SC-35 antigen. These observations strongly suggest that SC-35 is a splicing factor, but they do not provide direct proof. For example, SC-35 could be tightly associated with a splicing factor, but not be active itself. To address this possibility we have purified SC-35 to homogeneity by conventional and immunoaffinity chromatography methods (see Materials and methods). When purified SC-35 was fractionated on a SDS–polyacrylamide gel, only a 35 kDa doublet was observed (Figure 1A). This doublet is specifically recognized by αSC-35 antibodies in a Western blot (Figure 1B). Direct evidence that SC-35 is a splicing factor was provided by *in vitro* complementation experiments (Figure 1C and D). Extracts immunodepleted using the αSC-35 mAb showed no splicing activity (Figure 1C, lane 2 and D, lane 2). However, the splicing activity of the SC-35-depleted extracts could be reconstituted with purified SC-35 (Figure 1C, lanes 3 and 4). To show directly that the 35 kDa doublet is responsible for the splicing activity, we eluted the protein from a gel slice, and examined its ability to complement the immunodepleted extract. As shown in Figure 1D lane 3, splicing intermediates and products could be observed when the gel-eluted protein was added to the immunodepleted extract. In contrast, no splicing activity was detected in a mock elution of an adjacent gel slice (Figure 1D, lane 2). We conclude that SC-35 is an essential splicing factor.

Subnuclear localization of SC-35

The subnuclear distribution of SC-35 was examined in HeLa cells by immunofluorescent staining. The cells were stained with αSC-35, αSm and DAPI so that the distribution of

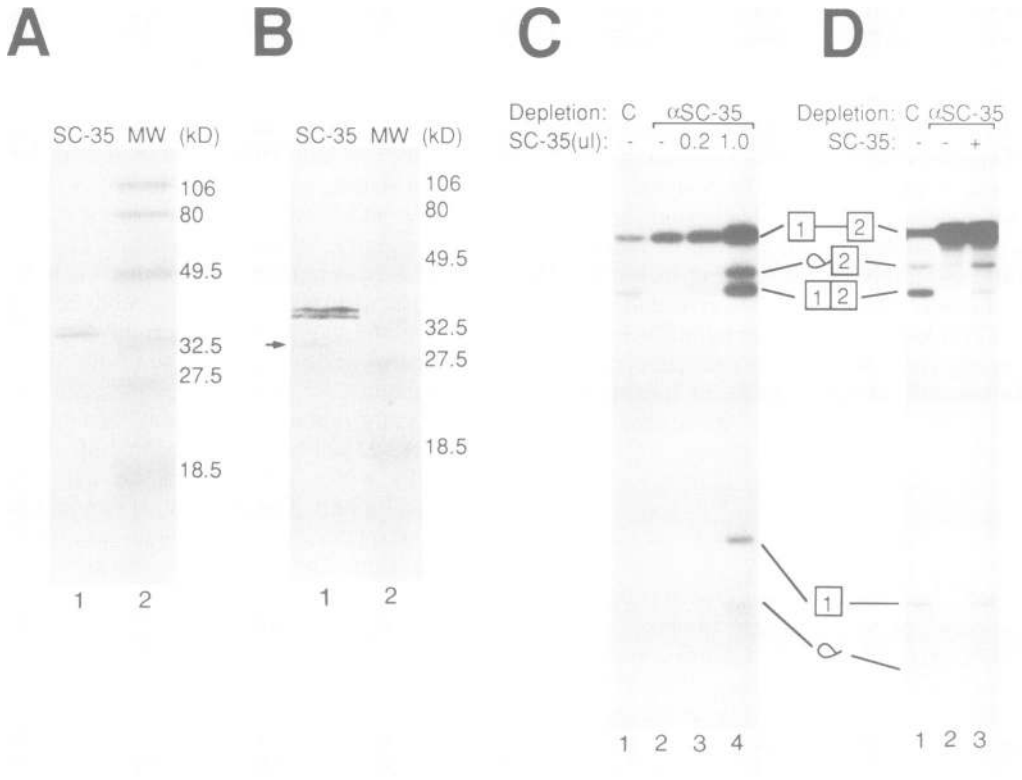

Fig. 1. SC-35 is an essential splicing factor. (**A**) One microliter of immunoaffinity-purified SC-35 run on a 12.5% SDS gel stained by Coomassie blue. (**B**) Western blotting of a parallel gel as (A) by αSC-35 mAb. Arrow indicates IgG light chain from αSC-35 affinity column. (**C**) Nuclear extracts were depleted with a control mAb or with αSC-35 as previously described (Fu and Maniatis, 1990) The SC-35-depleted extracts were then complemented with 0.2 or 1 μl of purified SC-35 for splicing of human β-globin pre-mRNA. (**D**) Purified SC-35 was eluted from SDS gel, renatured, and used to complement SC-35-depleted extracts. The decreased amount of surviving substrate in panel D is due to partial renaturation of gel isolated SC-35. MW: pre-stained low range protein standards (Bio-Rad).

SC-35 could be compared with that of snRNP antigens and DNA in the same cells. As previously demonstrated in human MG63 osteosarcoma cells (Fu and Maniatis, 1990), the nuclear distribution of SC-35 appears as a speckled pattern that occupies a portion of the nucleoplasm excluding the nucleoli (Figure 2b). When cells are immunostained with αSm antibodies a speckled pattern is also observed (Figure 2c). This pattern is coincident with the SC-35 speckles (compare Figure 2b and c), but αSm antibodies also show more diffuse nuclear immunostaining. These immunostaining patterns were identical regardless of the fixation used (formaldehyde, glutaraldehyde, methanol) and even in cells which were not fixed prior to immunolabeling (data not shown).

The three-dimensional distribution of SC-35 was examined by confocal laser scanning microscopy. Figure 3a is a red–green stereo pair showing a group of nuclei as seen from the top of the nuclei looking down toward the bottom of the nuclei. Figure 3b is a 180° rotation of these same nuclei now looking from the bottom of the nuclei up toward their top. These 3-D data show many connections between the SC-35 positive nuclear domains suggesting that SC-35 is organized in a network within the nucleoplasm. A similar three-dimensional distribution was previously described for snRNP antigens (Spector, 1990).

The subnuclear distribution of SC-35 and snRNP antigens was further compared by immunoelectron microscopy. HeLa cells were prepared by pre-embedding immunolabeling, and SC-35 was observed using peroxidase conjugated secondary antibodies (Figure 4). Immunostaining was confined to very distinct subnuclear regions (speckles) and each of these regions appeared as rather compact immunopositive zones (Figure 4a–b). The cytoplasm, nucleoli, and the background nucleoplasm did not show any immunoreactivity (Figure 4a–d). Examination of numerous electron micrographs showed the immunostained regions come into

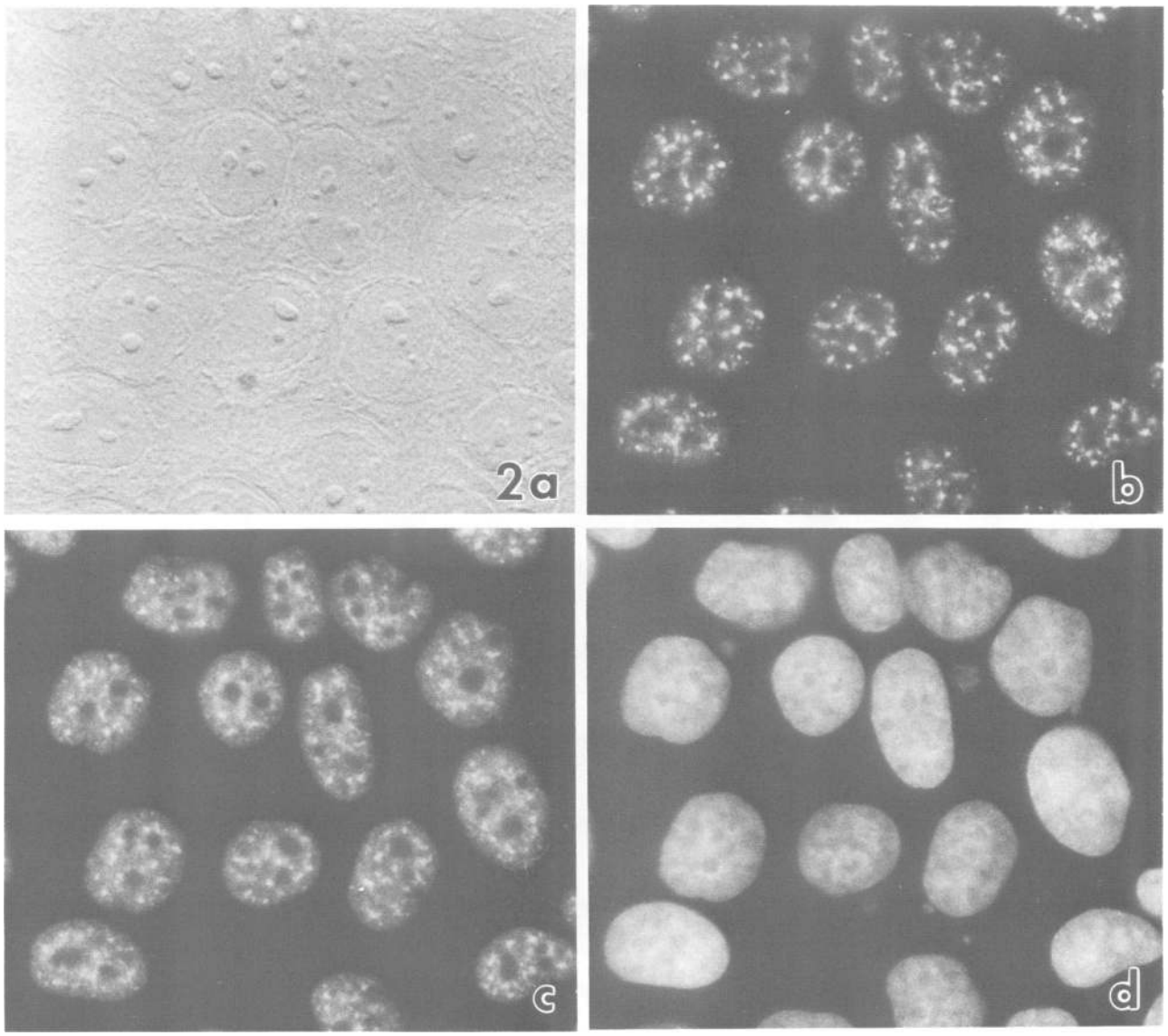

Fig. 2. Triple label fluorescence showing the localization of SC-35 (**b**), snRNP antigens (**c**) and DNA (**d**) in a population of HeLa cells. SC-35 and snRNP antigens colocalize in a speckled nuclear distribution pattern (compare b and c). In addition to the speckled pattern a subset of snRNP antigens are also somewhat more diffusely distributed in the nucleoplasm (c). (**a**) is a differential interference contrast image of the cell population. 1000×.

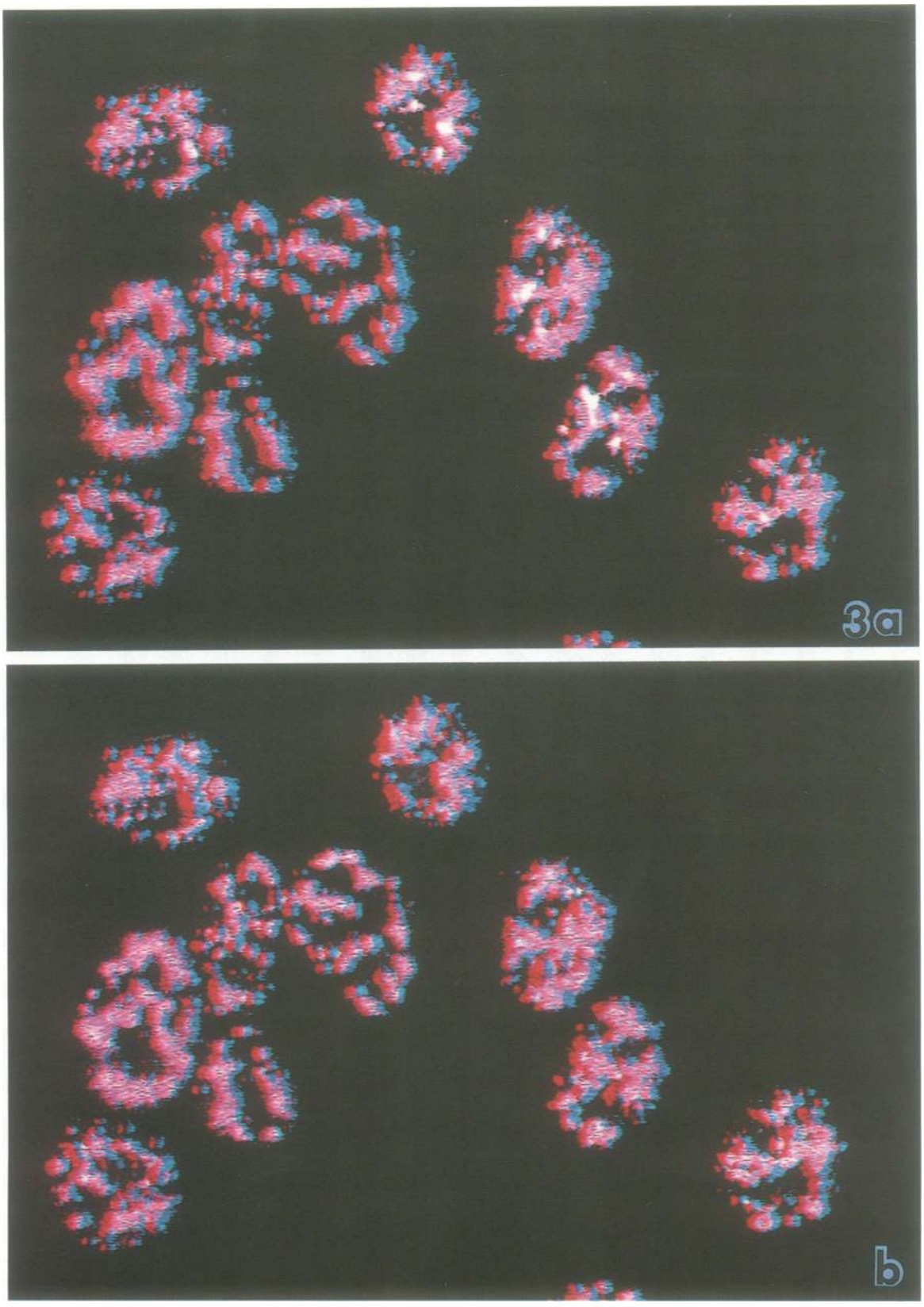

Fig. 3. Red–green stereo images showing the 3-D distribution of SC-35 in 10 HeLa cells. These pseudo-colored images were generated using a confocal laser scanning microscope. Each data set was generated from 25, 250 nm optical sections. When observed with red–green stereo glasses SC-35 is observed to be arranged in a network-like distribution pattern. Image (**a**) is looking from the top of the nuclei down toward the bottom of the cells and image (**b**) is a 180° rotation of the same group of cells looking from the bottom of the nuclei up toward the top of the cells. 1800×.

contact with the nuclear lamina-envelope (Figure 4c) and the nucleolar surface (Figure 4d). Occasionally, the point of attachment to the nucleolar surface appeared as a distinct structure. However, standard electron microscopy failed to reveal an identifiable substructure on the nucleolar surface. When HeLa cells are stained with αSm antibodies a speckled

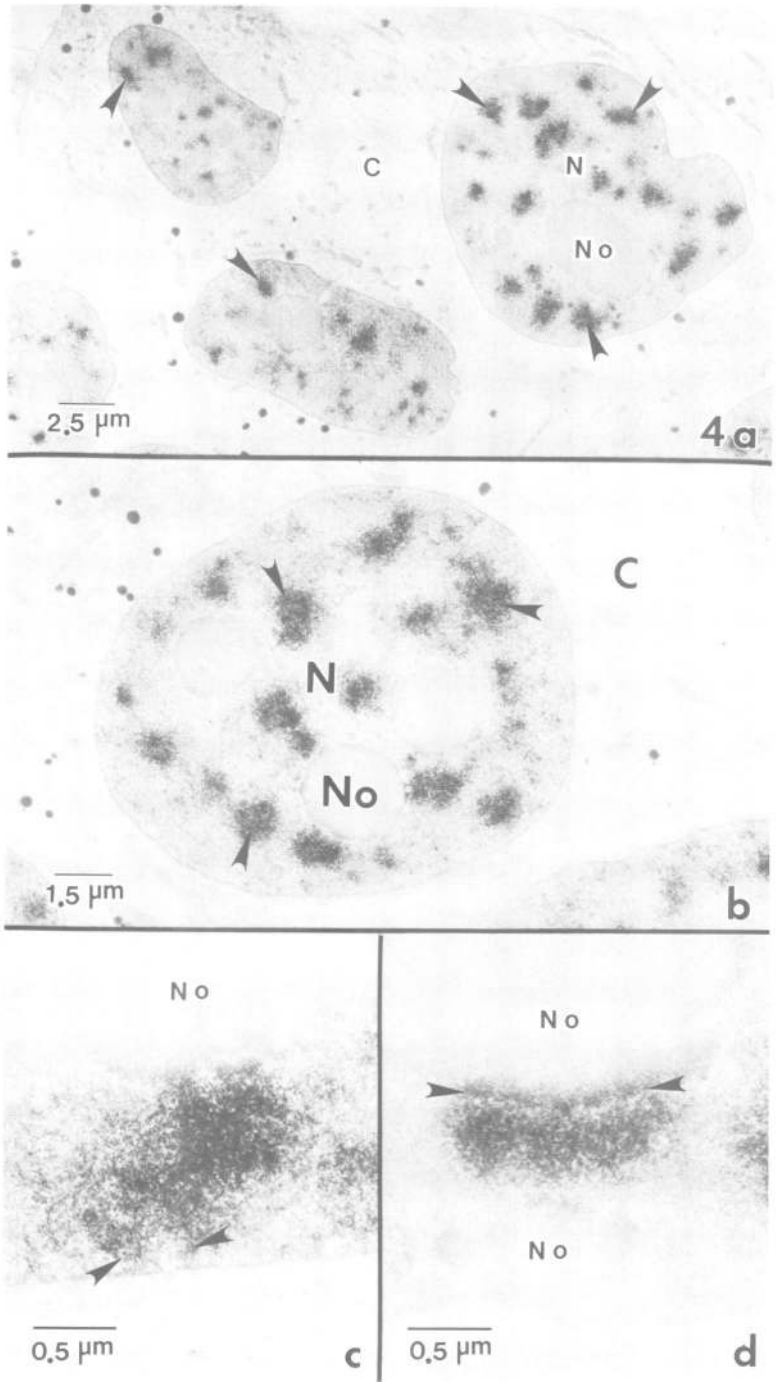

Fig. 4. Immunoelectron microscopic distribution of SC-35. (**a**) Low magnification view showing several immunostained cells. The protein distribution is observed by immunoperoxidase staining (arrowheads). SC-35 appears in clusters within the nucleoplasm (N); it is not present in the nucleoli (No) or in the cytoplasm (C) (**b**) Regions of SC-35 immunostaining extend out to the nuclear lamina-envelope (**c**, arrowheads) and come into direct contact with the nucleolus (No) (**d**, arrowheads).

immunostaining pattern was also observed (Figure 5a). While both snRNP antigens and SC-35 colocalize within the speckled regions, when observed by immunoelectron microscopy αSC-35 antibodies appear to immunostain these regions more intensely (Figure 4b) than do αSm (Figure 5a) or αU1 (unpublished data) antibodies. In addition to the speckled distribution of snRNP antigens, some diffuse immunoreactivity was also observed (Figure 5a). The diffuse staining does not result from non-specific binding of secondary antibody, because cells incubated only with peroxidase conjugated secondary antibodies did not show any immunoreactivity (Figure 5b). We conclude that SC-35 is localized to a subset of snRNP positive nuclear domains.

SC-35 is associated with interchromatin granules and perichromatin fibrils

In order to determine precisely the subnuclear localization of SC-35 and to compare its distribution with previously identified subnuclear structures, immunocytochemistry was performed on cell sections. Sections were immunolabeled with αSC-35 followed by colloidal gold conjugated secondary antibodies. After antibody staining, sections were

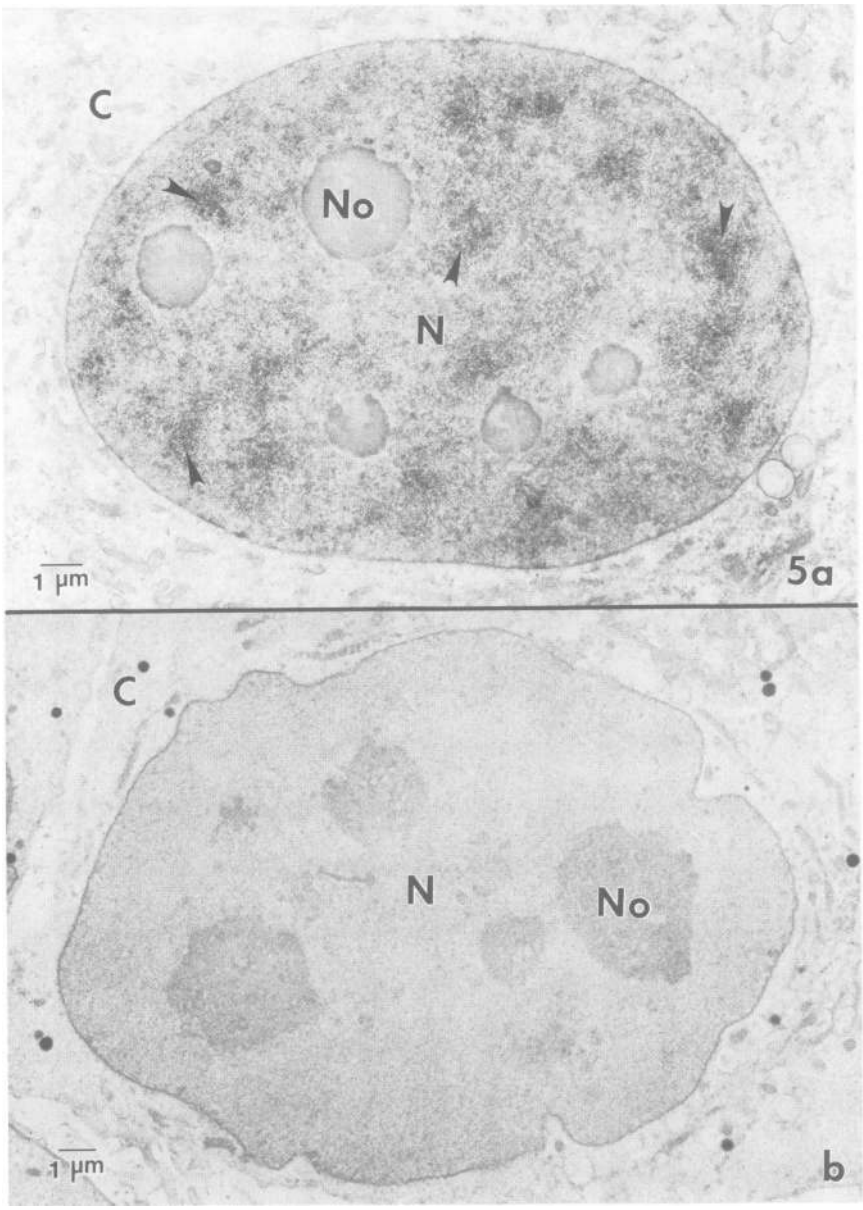

Fig. 5. Cells immunostained to localize snRNP antigens show a speckled distribution pattern in addition to some level of diffuse immunostaining within the nucleoplasm (**a**). Control cells immunostained with secondary antibody only show no immunostaining within the cell nucleus (**b**).

post-stained using the EDTA-regressive method (Bernhard, 1969). This staining method provides differential contrast to RNA-containing cell structures, while DNA-containing structures appear less stained. When such preparations were examined, it became clear that, while areas of condensed chromatin, nucleoli, and the cytoplasm exhibit few to no colloidal gold particles, SC-35 is localized to clusters of interchromatin granules and to perichromatin fibrils (Figure 6). SnRNPs have been previously described to be associated with one or more of these nuclear components (Spector *et al.*, 1983; Fakan *et al.*, 1984; Puvion *et al.*, 1984). The immunolabeled RNP-containing structures appear to form a network or reticulum in the nucleoplasm (Figure 6a) similar to what we have previously reported for the distribution of snRNP antigens using 3-D reconstruction techniques (Spector, 1990). Others have identified a ribonucleoprotein network or interchromatin net in 2-D images using cytochemical staining (Puvion and Bernhard, 1975) or nuclear matrix preparations (Smetana *et al.*, 1963). Here, we demonstrate for the first time that a specific non-snRNP splicing factor is indeed present in these structures.

Localization of SC-35 and snRNP antigens have different nuclease and heat shock sensitivities

Since both snRNP antigens and SC-35 are colocalized within interchromatin granules and perichromatin fibrils, we were interested in determining the molecular basis for the associations between these splicing factors and nuclear structures. HeLa cells were digested with either DNase I (Figure 7a–d) or RNase A (Figure 7e–h) prior to immunolocalization of SC-35, snRNP antigens and DNA. Cells digested with DNase I (Figure 7a–d) showed no alteration in the distribution of SC-35 (7b) or snRNP antigens (7c). To ensure that DNA had been digested, cells were monitored by DAPI staining (Figure 7d) and did not exhibit any fluorescence with this DNA-specific fluorochrome

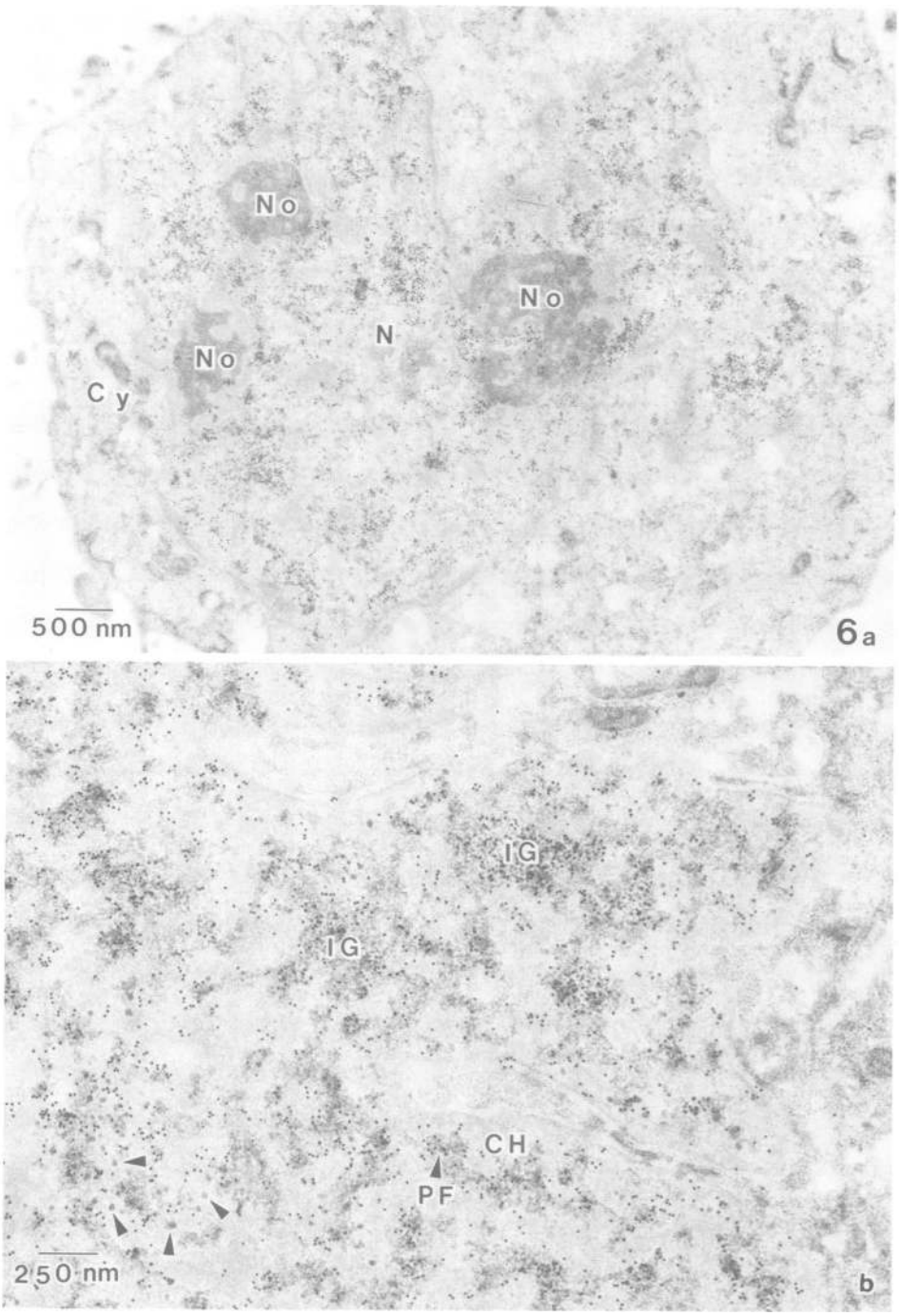

Fig. 6. Immunogold labeling of interchromatin granules and perichromatin fibrils by SC-35 antibody. (**a**) Low magnification view of a HeLa cell nucleus showing colloidal gold immunolabeling of SC-35 over the RNP network. (**b**) High magnification of a portion of another nucleus showing regions of interchromatin granules (IG) and perichromatin fibrils (PF) to be immunolabeled. Cytoplasm (Cy), nucleoli (No), chromatin (Ch) and perichromatin granules (arrowheads) are not immunolabeled. Post-stained by the EDTA-regressive method (Bernhard, 1969).

(Figure 7d). RNase A digestion also resulted in no change in the distribution of SC-35 (Figure 7f). However, RNase A digestion did result in an alteration in the distribution of snRNP antigens in the same cells (Figure 7g). The brightly fluorescent regions observed in undigested cells immunolabeled with αSm antibodies (Figure 2c) were greatly reduced in fluorescence intensity or totally absent (Figure 7g). SnRNP antigens appeared to be diffusely distributed throughout the nucleoplasm excluding the nucleoli (Figure 7g). RNase A digestion had no effect on the distribution of DNA (Figure 7h). Thus, both SC-35 and snRNP antigens are present within the same nuclear region, but their localization involves different molecular interactions. Moreover, these results show that the existence of the speckled regions is not dependent upon the presence of snRNP antigens.

Heat shock was previously shown to inhibit pre-mRNA processing in *Drosophila* (Yost and Lindquist, 1986) and mammalian (Bond, 1988) cells. We were therefore interested in the effect of heat shock on the distribution of splicing

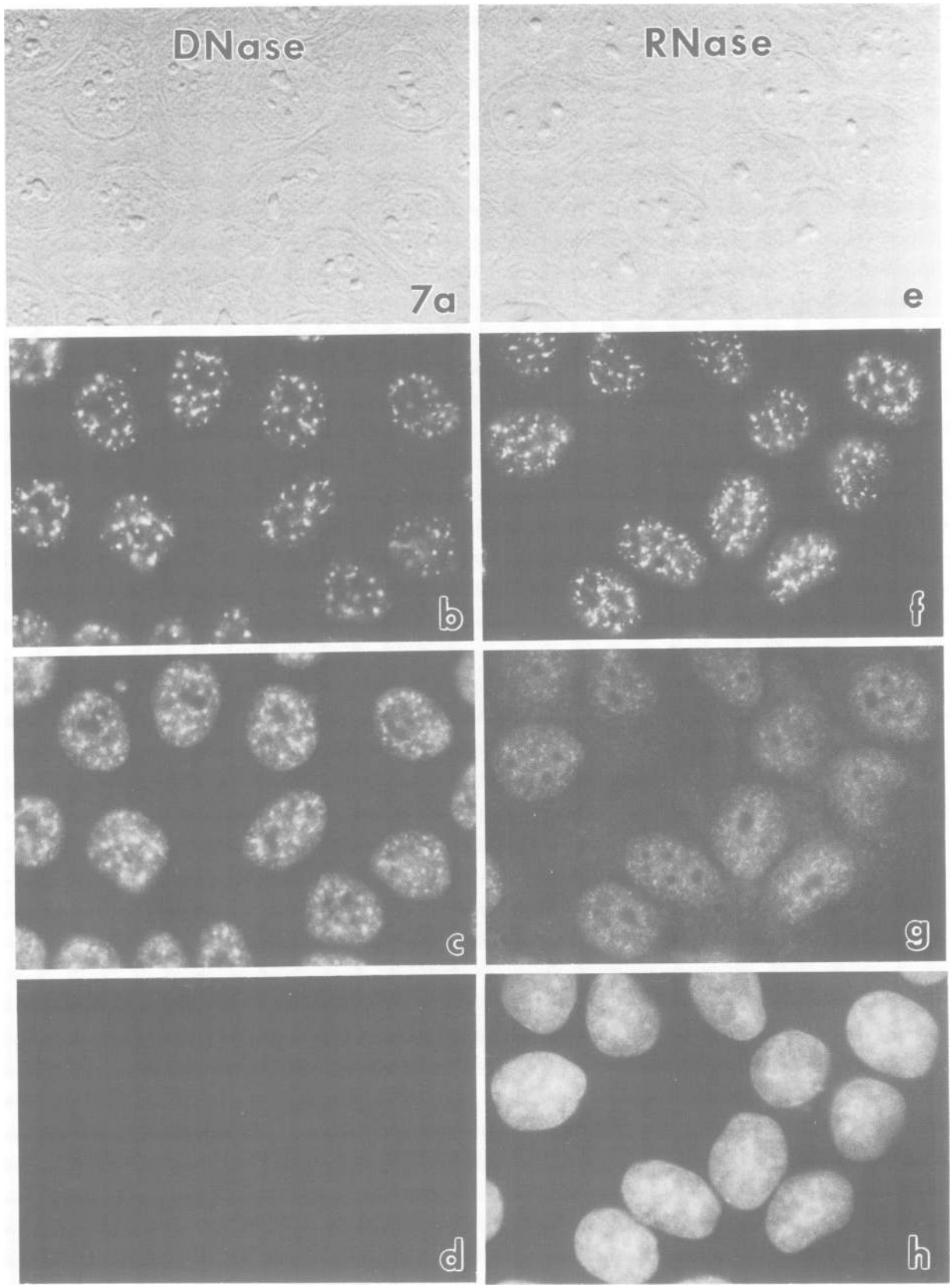

Fig. 7. Comparative nuclease sensitivity of SC-35 and snRNP antigen distribution patterns. Cells were treated with DNase and triple labeled to examine the distribution of SC-35, snRNP antigens and DNA (DAPI staining) (**b**–**d**). DNase digestion has no effect on the localization of SC-35 (**b**) or snRNP antigens (**c**); however, DNA is completely removed from the cell nuclei (**d**). Cells treated with RNase A (**f**–**h**) show no change in the distribution of SC-35 (**f**) or DNA (**h**); however, the speckled distribution of snRNP antigens is significantly reduced and some snRNP antigen immunostaining appears to be diffusely distributed throughout the nuclei (**g**). 1000×.

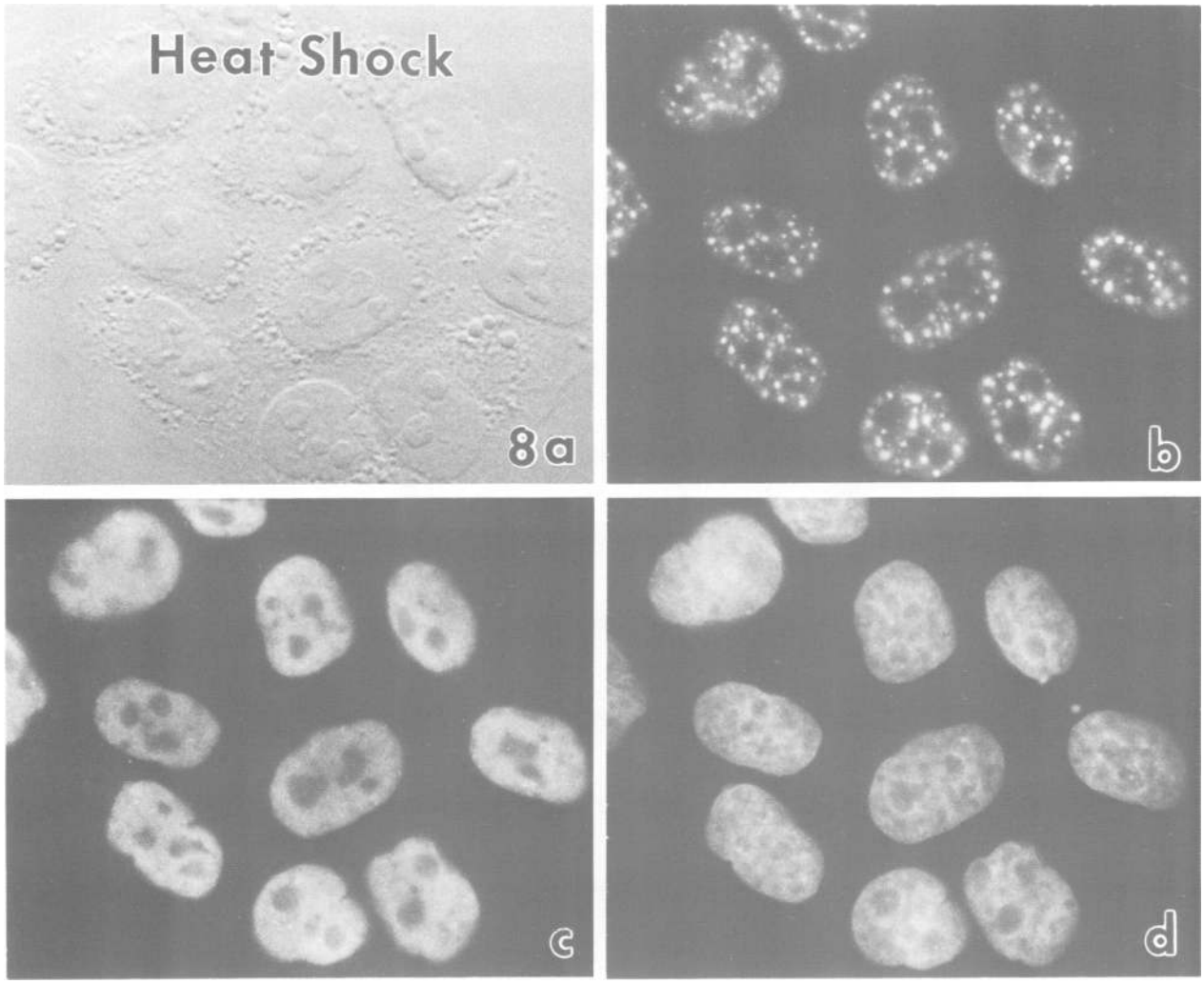

Fig. 8. Upon heat shock (45°C· 15 min) there is a slight change in the distribution pattern of SC-35. While the speckled pattern of SC-35 appears to show connections in control cells (Figure 2b), after heat shock the speckles appear more rounded and connections between speckles are not apparent (**b**). In the same cells after heat shock snRNP antigens appear to be uniformly distributed throughout the nucleoplasm (**c**), excluding the nucleoli, rather than being present in speckles. 1500×.

factors. HeLa cells were heat shocked at 45°C for 15 min and the localizations of SC-35, snRNP antigens and DNA investigated by fluorescence microscopy. Heat shock appeared to have a differential effect on the distribution of SC-35 and snRNP antigens. After heat shock the clusters enriched in SC-35 appeared to be more rounded (Figure 8b) than in control cells (Figure 2b), and the connections between clusters in control cells (Figure 2b) were not evident in heat shocked cells (Figure 8b). In contrast to the subtle change in the distribution of SC-35 after heat shock, a dramatic change was observed in the distribution of snRNP antigens (Figure 8c). SnRNP antigens appeared to be uniformly distributed throughout the nucleoplasm excluding the nucleoli after heat shock (Figure 8c). The distribution of DNA was not affected by heat shock in these cells (Figure 8d). Thus, heat shock did not result in a disruption of the speckled domain, instead, it affected the association of snRNP antigens with these nuclear regions.

Splicing components are differentially localized during mitosis

Previous studies demonstrated that snRNP antigens undergo reorganization during mitosis (Reuter *et al.*, 1985; Spector and Smith, 1986; Verheijen *et al.*, 1986; Leser *et al.*, 1989). We therefore examined the distribution of SC-35 during mitosis and compared its reorganization with that of snRNP antigens and DNA. As cells enter prophase, prior to nuclear envelope breakdown, the chromosomes begin to condense (Figures 9d and 10a) and both snRNP antigens (Figure 9c) and SC-35 are more diffusely distributed (Figures 9b and 10a) than in interphase. In addition, a small amount of SC-35 immunoreactivity is detected in the cytoplasm (Figures 9b and 10a). This cytoplasmic staining may be due to leakiness of the nuclear envelope prior to its breakdown later in prophase, and the disorganization of the snRNP antigen localization pattern (Figure 9b). During metaphase the chromosomes are maximally condensed, and they align along the equatorial plane of the spindle (Figures 9h and 10b). At this phase of mitosis, both SC-35 (Figures 9f and 10b) and snRNP antigens (Figure 9g) are detected as diffuse staining throughout the cytoplasm (Figure 9). During late anaphase, when chromosomes arrive at opposite poles, SC-35 begins to reorganize into speckles (Figures 9j and 11a) while snRNP antigens remain diffusely distributed throughout the cytoplasm (Figure 9k). When the chromosomes decondense in telophase, the nuclear envelope is reformed and SC-35

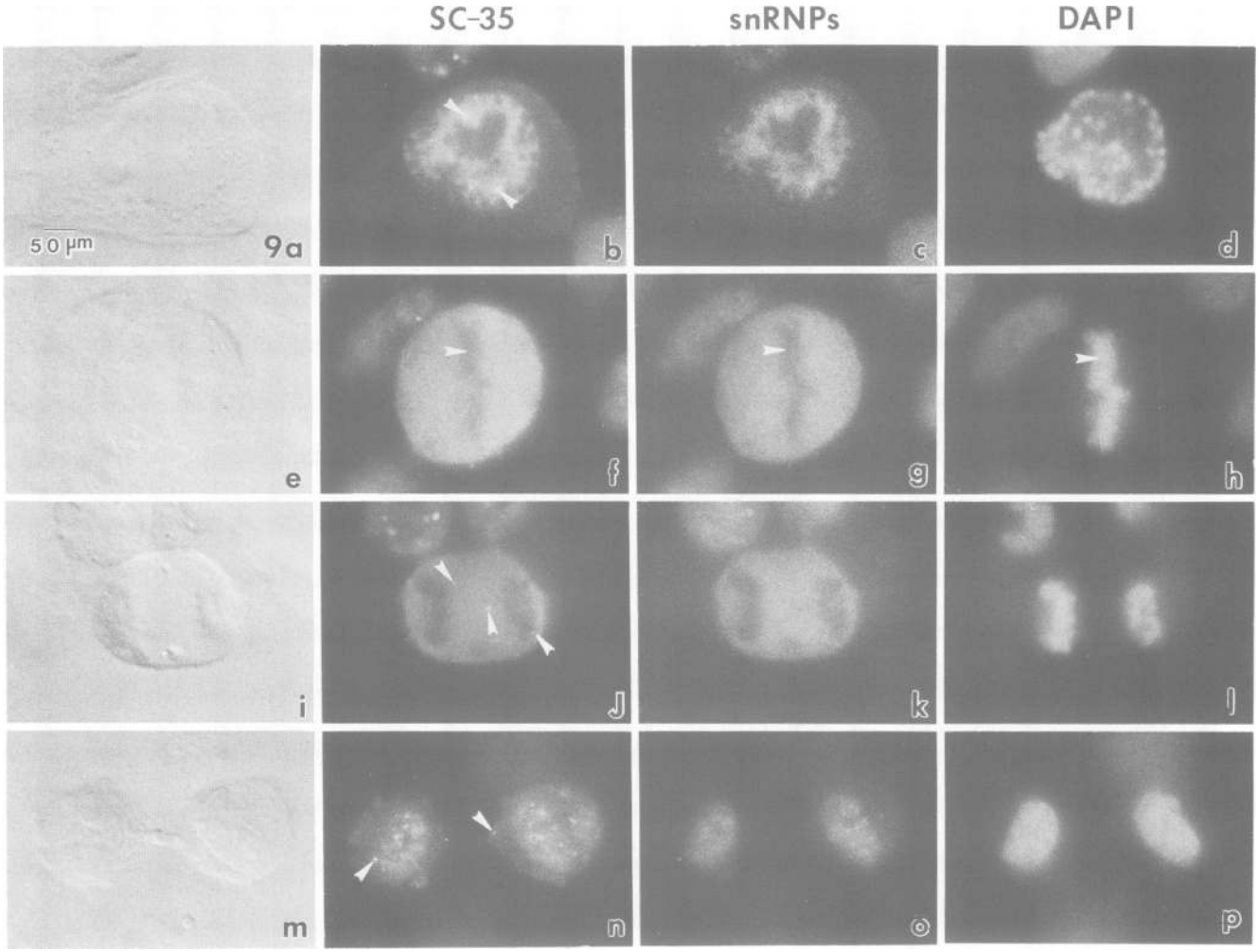

Fig. 9. Redistribution of SC-35, snRNP antigens and DNA during mitosis. As cells enter prophase (**a**–**d**) most of the speckles break up, SC-35 (**b**) and snRNP antigens (**c**) are more uniformly distributed between the condensing chromosomes (seen by immunofluorescence in **d**). In addition, some diffuse immunoreactivity appears to be present in the cytoplasm of prophase cells (b). During metaphase (**e**–**h**) both SC-35 (**f**) and snRNP antigens (**g**) are uniformly distributed throughout the cytoplasm. However, these antigens are not associated with the interior of the chromosomes in the metaphase plate (arrowheads). During anaphase (**i**–**l**) SC-35 begins to reassociate into speckles (**j**, arrowheads) while snRNP antigens are still uniformly distributed throughout the cytoplasm (**k**). During telophase (**m**–**p**) the nuclear envelope is reformed and while all of the snRNP immunoreactivity is contained within the nucleus (**o**) in many cells, several SC-35 speckles (**n**, arrowheads) appear in the cytoplasm in addition to the typical nuclear immunostaining. 2000×.

(Figures 9n and 11b) and snRNP antigen (Figure 9o) clusters are present within the newly formed daughter nuclei. However, some SC-35 clusters appear to remain in the cytoplasm after the nuclear envelope is reformed (Figures 9n and 11b). In contrast, snRNP antigen-containing clusters are never detected in the cytoplasm of telophase cells. These observations suggest that localization of SC-35 in clusters during anaphase may be part of nuclear matrix reorganization, while snRNP antigen association is a relatively later event, associated with the assembly of splicing components within the speckled regions on the surface of decondensing chromosomes in telophase.

Discussion

An understanding of the molecular organization of the mammalian cell nucleus could provide insights into the mechanisms involved in coordinating RNA transcription, processing and transport. In the present studies, we systematically investigated the nuclear distribution of snRNP antigens and a non-snRNP splicing factor, SC-35. Although these essential splicing components have similar nuclear distributions, they display differences in their nuclear associations and behavior during mitosis.

A significant difference between the localization of SC-35 and snRNP antigens was previously demonstrated by light microscopy (Fu and Maniatis, 1990) and confirmed here by electron microscopy. While SC-35 is restricted to the speckled regions of the nucleus, snRNP antigens are concentrated in the speckled regions but are also diffusely distributed throughout the nucleus. This diffuse nuclear staining may represent an excess soluble population of snRNPs, it may represent snRNPs in transit to snRNP clusters from their assembly sites in the cytoplasm (Zieve and Sauterer, 1990), or it may correspond to snRNPs that have moved from the clusters to other nuclear regions containing nascent pre-mRNA. Similar to previous studies on the localization of snRNP antigens (Spector, 1990), the speckled nuclear distribution of the SC-35 protein appears to form a network in the cell nucleus.

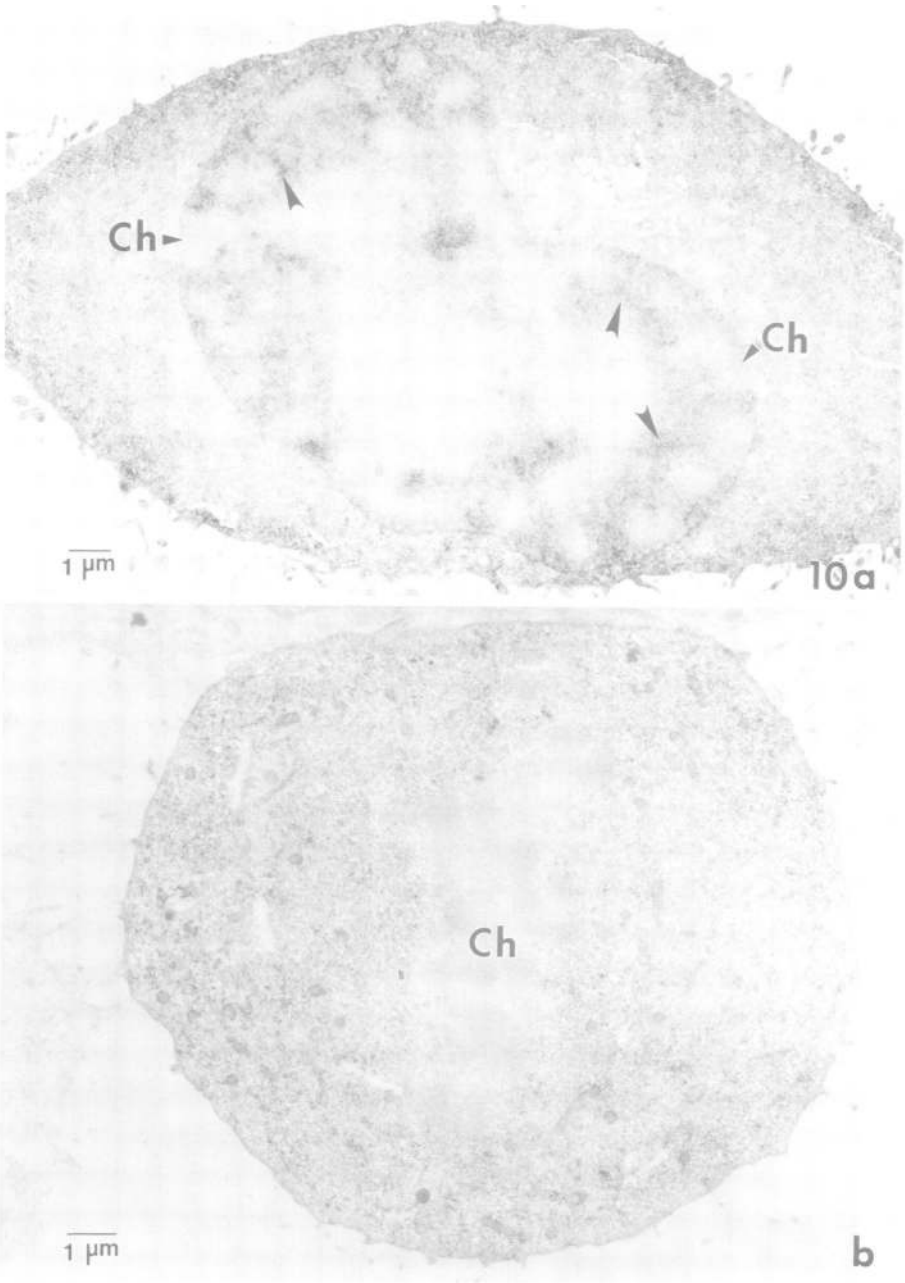

Fig. 10. Immunoelectron microscopic localization of SC-35 during prophase (**a**) and metaphase (**b**). During prophase the immunostaining pattern of SC-35 seems to be redistributed (arrowheads) between the condensing chromosomes (Ch). In addition some SC-35 immunoreactivity appears to leak out of the nucleus and be present within the cytoplasm. During metaphase (**b**) SC-35 appears to be uniformly distributed throughout the cytoplasm, however, the internal regions of the chromosomes (Ch) are devoid of immunoreactivity.

Nuclear localization of SC-35 and snRNP antigens is differentially sensitive to RNase digestion and heat shock

Differences in the effects of RNase digestion and heat shock on the nuclear localization of snRNP antigens and SC-35 suggest that the two types of splicing components are localized through distinct intermolecular interactions. While DNase I digestion does not affect the localization of either component, RNase A digestion alters the distribution of snRNP antigens, but not SC-35. These data suggest that snRNP antigens are associated with the speckled region through RNA−RNA or RNA−protein interactions, while SC-35 is associated with the speckled region through protein−protein interactions.

Heat shock has a relatively small effect on the localization of SC-35. The speckles detected with αSC-35 antibodies became more rounded or compact in appearance upon heat shock, and the connections between speckles were no longer apparent. In contrast, the association of snRNP antigens with the speckles was completely disrupted by heat shock. These findings are of particular interest in light of earlier biochemical studies showing that treatment of cells with heat shock blocks RNA processing (Yost and Lindquist, 1987; Bond, 1988). In addition, splicing activity was shown to be abolished in HeLa cell nuclear extracts prepared from cells exposed to a severe heat stress (Bond, 1988; Shukla *et al.*, 1990). Moreover, the U4/U5/U6 particle (Bond, 1988; Shukla *et al.*, 1990) and the U2 particle (Bond, 1988) are

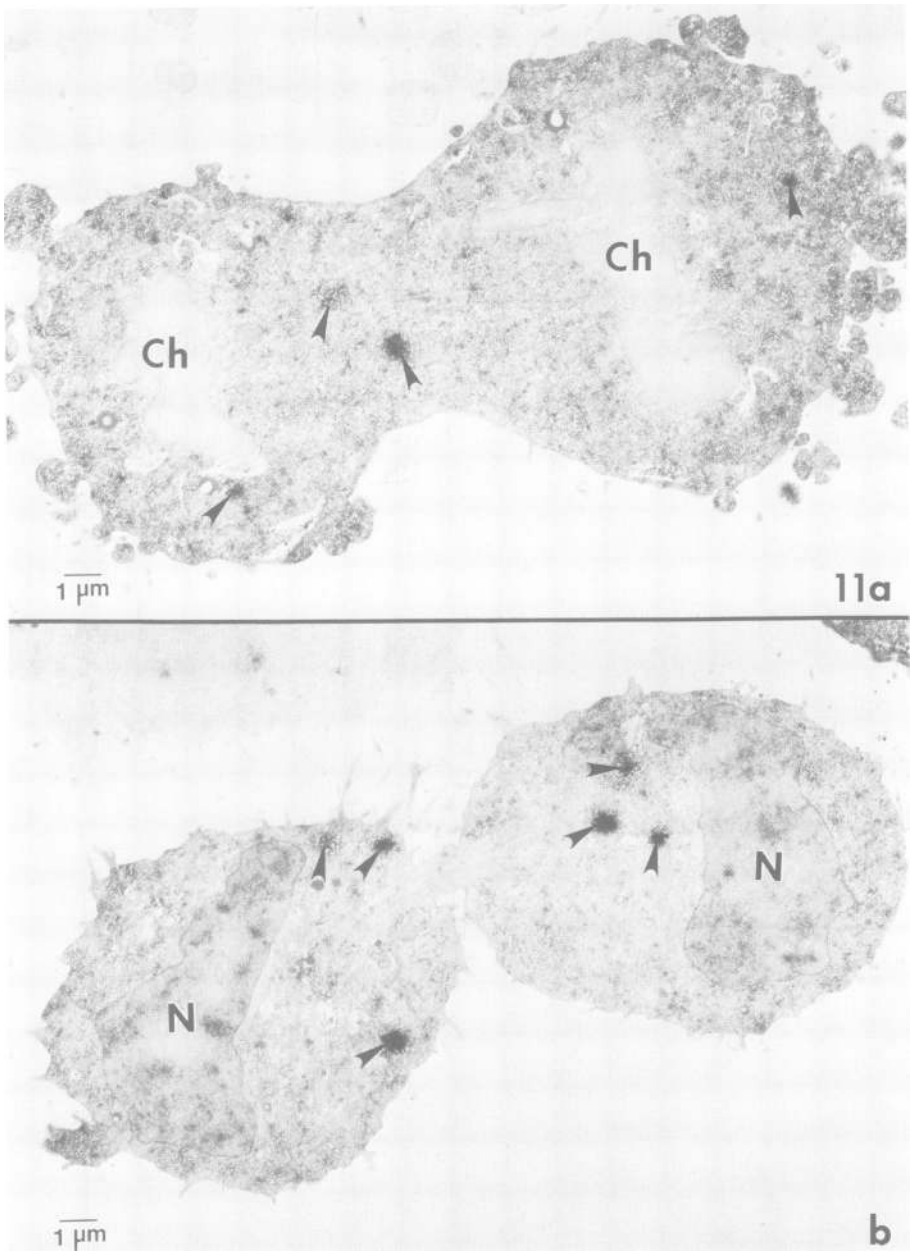

Fig. 11. Immunoelectron microscopic localization of SC-35 during anaphase (**a**) and telophase (**b**). During anaphase clusters of SC-35 begin to reform within the cytoplasm (arrowheads). In addition, the protein is distributed throughout the cytoplasm. During late telophase the chromosomes decondense, the nuclear envelope is reformed, and a speckled pattern of SC-35 reforms within each daughter nucleus (N). In addition, several speckled clusters of SC-35 which were not incorporated into the nucleus are present within the cytoplasm of telophase cells (arrowheads).

disrupted in heat shocked cells. Finally, a dramatic decrease of αSm fluorescence was observed in sections of heat shocked *Drosophila* embryos, and this decrease was paralleled by a loss of splicing activity (Wright-Sandor *et al.*, 1989). Thus, heat shock appears to disrupt spliceosomes, but does not dramatically affect the association of SC-35 with subnuclear regions.

Previous studies have shown that SC-35 is more tightly associated with the nucleus than snRNPs. Western blotting studies with αSC-35 and αSm antibodies have shown that both antigens are present in extracts prepared from nuclei. However, S100 extracts, which contain significant amounts of Sm antigens and snRNPs, do not contain SC-35 (Fu and Maniatis, 1990). In addition, we find that SC-35 remains bound to the nuclear matrix in the presence of high salt, conditions under which all of the snRNPs are released (X.-D.Fu and T.Maniatis, unpublished results). Matrix preparations were previously shown to contain pre-mRNA (Ciejek *et al.*, 1982; Mariman *et al.*, 1982), splicing components (Zeitlin *et al.*, 1987), snRNPs (Vogelstein and Hunt, 1982; Spector *et al.*, 1983; Smith *et al.*, 1986), functional spliceosomes (Zeitlin *et al.*, 1989) and a putative splicing factor (Smith *et al.*, 1989). The unusually tight association of SC-35 with the nuclear matrix suggests that

this splicing factor may be involved in interactions between spliceosomes and the matrix.

Differences in the timing of assembly and disassembly of SC-35 and snRNP antigens into speckled regions during mitosis

Our analysis of the formation of speckled regions during mitosis revealed significant differences between snRNP antigens and SC-35. In previous studies, both snRNP proteins and snRNAs have been shown to redistribute during mitosis (Reuter *et al.*, 1985; Spector and Smith, 1986; Verheijen *et al.*, 1986: Leser *et al.*, 1989). We find that snRNP antigens and SC-35 redistributed in a similar manner during prophase and metaphase, but a dramatic difference was observed during the later phases of mitosis. Late in anaphase when daughter chromosomes were almost at the poles SC-35 began to reform speckles within the cytoplasm. In contrast, snRNP antigens were diffusely distributed at this stage of mitosis. Thus, the assembly of SC-35 into concentrated speckled regions is initiated prior to the association of snRNP antigens with the speckled regions. A similar distribution has been reported for a nuclear matrix-associated protein (Smith *et al.*, 1985) which is thought to be a splicing factor (Smith *et al.*, 1989). We have not observed snRNP-containing mitotic granule clusters in metaphase and anaphase cells as has previously been reported (Leser *et al.*, 1989). Once the nuclear envelope reforms in late telophase, a speckled distribution pattern reforms in each daughter nucleus and both SC-35 and snRNP antigens are once again colocalized within speckled nuclear domains.

In summary, the splicing factor SC-35 is tightly bound to the nuclear matrix, and its association with the nucleus is resistant to DNase, RNase and heat shock. In addition, SC-35 is one of the earliest known markers for the assembly of speckled regions during mitosis. These observations suggest that SC-35 not only plays an important role in the earliest steps of pre-mRNA splicing (Fu and Maniatis, 1990), it may also be involved in the assembly and attachment of splicing components to the nuclear matrix.

Possible implications of the subnuclear localization of SC-35

At the electron microscopic level, SC-35 (this paper) and snRNPs (Fakan *et al.*, 1984; Puvion *et al.*, 1984) localize to nuclear regions enriched in interchromatin granules and perichromatin fibrils. The interchromatin granule regions contain particles with a mean diameter of 200–250 Å which are linked together by thin fibrils (Monneron and Bernhard, 1969). Perichromatin fibrils are found at the periphery of regions of condensed chromatin and dispersed throughout the interchromatin space (Fakan and Puvion, 1980). These fibrils have a diameter of 30–50 Å but can measure up to 200 Å in diameter. *In situ* autoradiographic studies following [^{3}H]uridine incorporation have shown little to no labeling over interchromatin granule clusters (Fakan and Bernhard, 1971; Fakan and Nobis, 1978). Based on these data and other studies interchromatin granule clusters are thought to contain RNA species with a slow turnover rate (for a review see Fakan and Puvion, 1980). These studies are consistent with the findings of snRNA-associated snRNP antigens within these nuclear regions (Spector *et al.*, 1983; Fakan *et al.*, 1984; Puvion *et al.*, 1984). In contrast to interchromatin granules, perichromatin fibrils are rapidly labeled with [^{3}H]uridine, suggesting that they correspond to nascent pre-mRNA (Bachellerie *et al.*, 1975; Fakan *et al.*, 1976). In fact, Monneron and Bernhard (1969) first suggested a relationship between these fibrils and extranucleolar RNA synthesis. Both snRNP and hnRNP antigens have been localized to these fibrils (Fakan *et al.*, 1984; Puvion *et al.*, 1984). Thus, SC-35 and many snRNP antigens appear to be concentrated in two distinct compartments in the nuclei of mammalian cells: the interchromatin granules which do not appear to contain pre-mRNA, and perichromatin fibrils where pre-mRNA may be localized.

Recent studies of the localization of splicing components in amphibian germinal vesicles provide insights into the functional significance of SC-35 localization in somatic cells (Gall and Callan, 1989; Wu *et al.*, 1991; see Gall, 1991 for review). The nascent RNA chains comprising the loops of lampbrush chromosomes are uniformly stained with antibodies against hnRNP proteins, snRNPs and SC-35. Thus, the loops appear to be packaged into a ribonucleoprotein complex that includes all of the components of the spliceosome for which probes are available. Anti-spliceosome antibodies, including αSC-35, also stain large extrachromosomal particles designated snurposomes (Gall, 1991). Snurposomes have been divided into three classes, designated A, B and C. The A snurposomes vary in size from 1 to 4 μM in diameter and appear to contain exclusively U1 snRNPs, while the B snurposomes are 4 μM in diameter, and contain U1, U2, U4, U5 and U6, as well as SC-35. C snurposomes can be as large as 20 μM in diameter, and appear to consist of an aggregate of B snurposomes. Gall and his coworkers have proposed that spliceosome components may be pre-assembled into macromolecular complexes in the B snurposome, analogous to the assembly of ribosomes in the nucleolus (Wu *et al.*, 1991). The complexes assembled in the snurposomes, rather than free snRNPs or hnRNPs would then associate with nascent pre-mRNA.

An analogous model can be proposed for mammalian cells. Like the B snurposomes, interchromatin granule clusters may be sites of spliceosome assembly and/or storage. Splicing factors may then move from these sites to the perichromatin fibrils where nascent pre-mRNA is located. Alternatively, pre-mRNA may move to regions adjacent to, but not coincident with, interchromatin granule clusters where splicing may occur. Recent studies of the nuclear localization of pre-mRNA are consistent with this possibility (Wang *et al.*, 1991; S.Huang and D.L.Spector, submitted). First, microinjected rhodamine-labeled pre-mRNAs colocalize with snRNPs and SC-35 in the speckled regions in somatic cell nuclei (Wang *et al.*, 1991). RNAs lacking splicing signals fail to localize in this manner. This observation shows that the speckled regions contain factors capable of binding specifically to pre-mRNA containing functional splice sites, but does not prove that endogenous pre-mRNAs localize to the speckled regions. Second, endogenous, nascent c-*fos* pre-mRNA detected by *in situ* hybridization is found in two highly concentrated regions that directly contact, but do not completely overlap with the speckled staining pattern detected by αSC-35 antibodies (S.Huang and D.L.Spector, submitted). Thus, splicing may occur in a region which is adjacent to or within the speckled regions.

A different view of spliceosome localization was recently reported by Carmo-Fonseca *et al.* (1991a). *In situ* hybridization (Carmo-Fonseca *et al.*, 1991a) and microinjection (Carmo-Fonseca *et al.*, 1991b) experiments with antisense probes made of 2′-OMe RNA detected three to four intensely labeled regions, 'foci' in each nucleus, rather than speckles. These foci are also detected with an antiserum raised against a peptide of the splicing factor U2AF (Carmo-Fonseca *et al.*, 1991a; Zamore and Green, 1991). However, this antiserum also detects a highly diffuse pattern of nuclear localization. In contrast to the antisense snRNA probes, the αSC-35 antibody stains speckles, but not foci (Carmo-Fonseca *et al.*, 1991b; S.Huang and D.L.Spector, unpublished results). Although the results of Carmo-Fonseca *et al.* (1991a,b) are consistent with the possibility that the speckled regions detected by αsnRNP and αSC-35 antibodies do not contain snRNAs, it is equally likely that snRNAs are present in these regions, but they are not accessible for hybridization to the oligonucleotides. In fact, a speckled staining pattern can be observed in nuclei of cells hybridized with oligonucleotide probes when cells are prepared in the presence of KCl concentrations that are used to prepare splicing extracts (see Carmo-Fonseca *et al.*, 1991a, Figure 5c). An additional point is that amphibian snurposomes, which were shown to contain all of the spliceosomal snRNAs by *in situ* hybridization, are also specifically detected by αSC-35 antibodies (Wu *et al.*, 1991). This finding is consistent with the observation that the speckled staining patterns observed with αSC-35 and αsnRNP antibodies in mammalian cells extensively overlap (Fu and Maniatis, 1990; this paper). The possibility that the speckled staining pattern detected by αSC-35 antibody does not contain snRNAs therefore seems very unlikely.

In summary, nuclear localization studies described here and elsewhere have detected spliceosome components in at least three distinct nuclear structures: (i) perichromatin fibrils, regions of the nucleus which represent a portion of the speckled immunostaining pattern and may contain nascent pre-mRNA; (ii) interchromatin granules, structures that represent another portion of the speckled staining pattern and may represent spliceosome assembly sites; and (iii) foci, structures that appear to correspond to coiled bodies (D.L.Spector, G.Lark and S.Wang, in preparation; Fakan *et al.*, 1984) and contain high levels of snRNA and the splicing factor U2AF (Carmo-Fonseca *et al.*, 1991a). Additional experiments will be required to determine whether the failure to detect snRNA in speckles and SC-35 in foci is a consequence of a real differential localization of splicing components, or a result of differential accessibility of these structures to antisense and antibody probes. The localization of spliceosome components in different regions of mammalian nuclei is perhaps not surprising, considering the complex distribution of snRNPs and SC-35 in amphibian oocytes.

Materials and methods

Immunoaffinity purification of SC-35

HeLa cell nuclear extract (5 ml) was brought to an ammonium sulfate concentration of 50%, and SC-35 was found in the supernatant by Western blotting. The supernatant was loaded directly onto a 1 ml phenyl-sepharose column equilibrated in Buffer A (20 mM Tris pH 7.9, 0.1 M KCl, 5% glycerol, 0.2 mM EDTA) and aggregates were removed by Centricon-100 filtration. SC-35 was concentrated by Centricon-30 (Amicon). This partially purified SC-35 was then loaded onto a 0.5 ml αSC-35-affinity column, and the SC-35 antigen was eluted with 0.1 M triethylamine pH 11.5, followed by dialysis against the buffer described above containing 20% glycerol. Isolation of SC-35 from an SDS gel slice was carried out following the protocol described by Baeuerle and Baltimore (1988).

Cell culture

HeLa cells were grown on glass coverslips in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum. For heat shock experiments Petri dishes containing coverslips and pre-warmed medium (45°C) were floated on a 45°C water bath for 15 min prior to fixation for immunofluorescence microscopy.

Immunofluorescence microscopy

Cells were prepared for immunofluorescence microscopy according to previously published procedures (Spector and Smith, 1986). Anti-Sm autoantibody (Schrier *et al.*, 1982) was used at a dilution of 1:300 and anti-SC-35 monoclonal antibody (Fu and Maniatis, 1990) was used at a dilution of 1:50. Fluorescein isothiocyanate (FITC) conjugated goat anti-human IgG and Texas Red conjugated goat anti-mouse IgG (Cappel Laboratories) were used at a dilution of 1:30 for 1 h at 20°C. After immunostaining cells were counterstained with 4′,6-diamidino-2-phenylindole:2HCl (DAPI) at a concentration of 1 mg/ml. Coverslips were mounted onto glass slides in 90% glycerol, 10% PBS plus 4% (w/v) *n*-propyl gallate.

Confocal microscopy

Cells were immunostained as above and coverslips were mounted in Immunomount (Shandon Inc.) containing 1 mg/ml *p*-phenylenediamine as an anti-oxidant. 0.5 μm optical sections were obtained using a Zeiss confocal laser scanning microscope equipped with a 63/1.4 N.A. oil immersion lens, an argon ion laser (λ = 488 nm), and a HeNe laser (λ = 543 nm). Optical sections spanning the entire nucleus were reconstructed using a Zeiss program package and images were viewed as pseudo-colored red−green stereo pairs.

Nuclease digestion

Cells were fixed in methanol for 2 min at −20°C, rinsed in PBS (pH 7.4) and incubated in either DNase I (100 μg/ml in PBS + 5 mM $MgCl_2$, RNase free) or RNase A (100 μg/ml in PBS, DNase free) for 2 h at 25°C. After several washes in PBS cells were prepared for immunofluorescence microscopy as described above.

Immunoelectron microscopy

Pre-embedding. Cells were fixed in 2% formaldehyde plus 0.1% glutaraldehyde for 15 min at 20°C and prepared for immunoelectron microscopy according to published procedures (Spector, 1984; Spector and Smith, 1986). Briefly, fixed cells were washed in 0.5 mg/ml sodium borohydride, permeabilized in 0.2% Triton X-100 and washed in PBS (pH 7.4) containing 1% normal goat serum (NGS) prior to incubation in primary antibody for 1 h at 37°C. Cells were then extensively washed in PBS and incubated in peroxidase conjugated secondary antibodies at a dilution of 1:20 for 1 h at 37°C. The peroxidase reaction product was developed by incubation in 0.5% diaminobenzidine containing 0.01% H_2O_2 for 5 min at 20°C. Cells were then fixed in osmium tetroxide and prepared for electron microscopy by standard methods (Spector and Smith, 1986). Thin sections (100 nm) cut on a Reichert Ultracut E ultramicrotome with a Diatome diamond knife were examined with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

Post-embedding. Cells were pelleted and fixed in 2% formaldehyde plus 0.5% glutaraldehyde for 15 min at 20°C and prepared for immunoelectron microscopy according to published procedures (Potashkin *et al.*, 1990). Thin LR White sections (100 nm) were collected and grids were floated face down in Tris buffered saline (TBS) containing 20 mM Tris (pH 7.6), 150 mM NaCl, 20 mM sodium azide, 1.0% Tween-20 and 5% bovine serum albumin (BSA), and 5% normal goat serum (NGS) for 60 min. Sections were then incubated in αSC-35 diluted 1:60 in TBS minus BSA and NGS at 4°C overnight in a humidified chamber. The following morning, the grids were equilibrated to room temperature and then washed for 15 min in TBS. The grids were then transferred to colloidal gold conjugated goat anti-mouse IgG (15 nm gold particles, Janssen Life Sciences Products) diluted 1:50 in TBS for 1 h at room temperature. Prior to incubation the diluted colloidal gold-labeled antibody was spun in a microfuge for 2 min at room temperature. After incubation the grids were washed for 15 min in TBS, 10 min in water and counterstained with the EDTA-regressive staining method (Bernhard, 1969). Samples were examined at 75 kV in a Hitachi H-7000 transmission electron microscope.

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References

Ast,G., Goldblatt,D., Offen,D., Sperling,J. and Sperling,R. (1991) *EMBO J.*, **10**, 425-432.

Bachellerie,J.-P., Puvion,E. and Zalta,J.-P. (1975) *Eur. J. Biochem.*, **58**, 327-337.

Baeuerle,P.A. and Baltimore,D. (1988) *Cell*, **53**, 211-217.

Bernhard,W. (1969) *J. Ultrastruct. Res.*, **27**, 250-265.

Bindereif,A. and Green,M. (1990) *Genetic Engng*, **12**, 201-224.

Bond,U. (1988) *EMBO J.*, **7**, 3509-3518.

Carmo-Fonseca,M., Tollervey,D., Barabino,S.M.L., Merdes,A., Brunner,C., Zamore,P.D., Green,M.R., Hurt,E. and Lamond,A.I. (1991a) *EMBO J.*, **10**, 195-206.

Carmo-Fonseca,M., Pepperkok,R., Sproat,B.S., Ansorge,W., Swanson,M. and Lamond,A.I. (1991b) *EMBO J.*, **10**, 1863-1873.

Ciejek,E.M., Nordstrom,J.L., Tsai,M.-J. and O'Malley,B.W. (1982) *Biochemistry*, **21**, 4945-4953.

Fakan,S. and Bernhard,W. (1971) *Exp. Cell Res.*, **67**, 129-141.

Fakan,S. and Nobis,P. (1978) *Exp. Cell Res.*, **113**, 327-337.

Fakan,S. and Puvion,E. (1980) *Int. Rev. Cytol.*, **65**, 255-299.

Fakan,S., Puvion,E. and Spohr,G. (1976) *Exp. Cell Res.*, **99**, 155-164.

Fakan,S., Leser,G. and Martin,T.E. (1984) *J. Cell Biol.*, **98**, 358-363.

Fu,X.-D. and Maniatis,T. (1990) *Nature*, **343**, 437-441.

Gall,J.G. (1991) In Wassarman,P. (ed.), *Advances in Developmental Biochemistry*. in press.

Gall,J.G. and Callan,H.G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6635-6639.

Ge,H. and Manley,J.L. (1990) *Cell*, **62**, 25-34.

Krainer,A. and Maniatis,T. (1985) *Cell*, **42**, 725-736.

Krainer,A. and Maniatis,T. (1988) In Hames,B.D. and Glover,D.M. (eds), *Frontiers in Molecular Biology: Transcription and Splicing*. IRL press, Oxford, pp. 131-296.

Krainer,A., Conway,G.C. and Kozak,D. (1990) *Genes Dev.*, **4**, 1158-1171.

Krainer,A., Conway,G.C. and Kozak,D. (1990) *Cell*, **62**, 35-42.

Lerner,E.A., Lerner,M.R., Janeway,L.A. and Steitz,J.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2737-2741.

Leser,G.P., Fakan,S. and Martin,T.E. (1989) *Eur. J. Cell Biol.*, **50**, 376-389.

Mariman,E.C., van Eekelen,C.A.G., Reinders,R.J., Berns,A.J.M. and van Venrooij,W.J.(1982) *J. Mol. Biol.*, **154**, 103-119.

Monneron,A. and Bernhard,W. (1969) *J. Ultrastruct. Res.*, **27**, 266-288.

Nyman,U., Hallman,H., Hadlaczky,G., Pettersson,I., Sharp,G. and Ringertz,N.R. (1986) *J. Cell Biol.*, 102, 137-144.

Potashkin,J.A., Derby,R.J. and Spector,D.L. (1990) *Mol. Cell. Biol.*, **10**, 3524-3534.

Puvion,E. and Bernhard,W. (1975) *J. Cell Biol.*, **67**, 200-214.

Puvion,E. and Moyne,G. (1981) In Busch,H. (ed.), *The Cell Nucleus*. Academic Press, New York, Vol. VIII, pp. 59-115.

Puvion,E., Viron,A., Assens,C., Leduc,E.H. and Jeanteur,P. (1984) *J. Ultrastruct. Res.*, **87**, 180-189.

Raska,I., Ochs,R.L. and Salamin-Michel,L. (1990) *Electron Micros. Rev.*, **3**, 301-353.

Reed,R. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8031-8035.

Reuter,R., Appel,B., Bringmann,P., Rinke,J. and Luhrmann,R. (1984) *Exp. Cell Res.*, **154**, 548-560.

Reuter,R., Appel,B., Rinke,J. and Luhrmann,R. (1985) *Exp. Cell Res.*, **159**, 63-79.

Ruskin,B., Zamore,P.D. and Green,M.R. (1988) *Cell*, **52**, 207-219.

Schrier,W.H., Reddy,R. and Busch,H. (1982) *Cell Biol. Int. Rep.*, **6**, 925-932.

Shukla,R.R., Dominski,Z., Zwierzynski,T. and Kole.R. (1990) *J. Biol. Chem.*, **265**, 20377-20383.

Smetana,K., Steele,W.J. and Busch,H. (1963) *Exp. Cell Res.*, **31**, 198-201.

Smith,H.C., Spector,D.L., Woodcock,C.L.F., Ochs,R.L. and Bhorjee,J (1985) *J. Cell Biol.*, **101**, 560-567.

Smith,H.C., Ochs,R.L., Fernandez,E.A. and Spector,D.L.. (1986) *Mol. Cell. Biochem.*, **70**, 151-168.

Smith,H.C., Harris,S.G., Zillmann,M. and Berget,S.M. (1989) *Exp. Cell Res.*, **182**, 521-533.

Spector,D.L. (1984) *Biol. Cell*, **51**, 109-112.

Spector,D.L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 147-151.

Spector,D.L., Schrier,W.H. and Busch,H. (1983) *Biol. Cell*, **49**, 1-10.

Spector,D.L. and Smith,H.C. (1986) *Exp. Cell Res.*, **163**, 87-94.

Steitz,J.A., Black,D. L., Gerke,V., Parker,K.A., Kramer,A., Frendeway,D. and Keller,W. (1988) In Birsnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer Verlag, Berlin, pp. 115-154.

Verheijen,R., Kuijpers,H., Vooijs,P., van Venrooij,W. and Ramaekers,F. (1986) *J. Cell Sci.*, **86**, 173-190.

Vogelstein,B. and Hunt,B.F. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 1224-1232.

Wang,J., Cao,L.-G., Wang,Y.-L. and Pederson,T. (1991) *Proc. Natl. Acad. Sci. USA*, in press.

Wright-Sandor,L.G., Reichlin,M. and Tobin,S.L. (1989) *J. Cell Biol.*, **108**, 2007-2016.

Wu,Z., Murphy,C., Callan,H.G. and Gall,J.G. (1991) *J. Cell Biol.*, **113**, 465-483.

Yost,H.J. and Lindquist,S (1986) *Cell*, **45**, 185-193.

Zamore,P.D. and Green,M.R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9243-9247.

Zamore,P.D. and Green,M.R. (1991) *EMBO J.*, **10**, 207-214.

Zeitlin,S., Parent,A., Silverstein,S. and Efstratiadis,A. (1987) *Mol. Cell. Biol.*, **7**, 111-120.

Zeitlin,S., Wilson,R.C. and Efstratiadis,A. (1989) *J. Cell Biol.*, **108**, 765-777.

Zieve,G. and Sauterer,R.A. (1990) *Crit. Rev. Biochem. Mol. Biol.*, **25**, 1-46.

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Colocalization of SC-35, and U1 and U2 snRNAs to speckled nuclear regions has recently been demonstrated using 2'-OMe oligonucleotide antisense probes provided by A.Lamond (S.Huang and D.L.Spector, submitted). In this case hybridization was carried out for longer periods of time than previously reported (Carmo-Fonseca *et al.*, 1991a).