

Transcription-dependent Colocalization of the U1, U2, U4/U6, and U5 snRNPs in Coiled Bodies

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Abstract. We have recently shown that discrete foci are present in the nuclei of mammalian cells in which each of the U1, U2, U4/U6, and U5 snRNPs involved in pre-mRNA splicing, and the non-snRNP-splicing factor U2AF, are concentrated (Carmo-Fonseca, M., D. Tollervy, R. Pepperkok, S. Barabino, A. Merdes, C. Brunner, P. D. Zamore, M. R. Green, E. Hurt, and A. I. Lamond. 1991. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:195–206; Carmo-Fonseca, M., R. Pepperkok, B. S. Sproat, W. Ansorge, M. S. Swanson, and A. I. Lamond. 1991. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1863–1873). Here, we identify these snRNP-rich organelles as coiled bodies. snRNPs no longer concentrate in coiled bodies after cells are treated with the transcription inhibitors α -amanitin or actinomycin D. snRNP association with coiled bodies is also disrupted by heat shock. This indicates that the association of

snRNPs with coiled bodies may be connected with the metabolism of nascent transcripts. A novel labeling method is described which shows both the RNA and protein components of individual snRNPs colocalizing in situ. Using this procedure all spliceosomal snRNPs are seen distributed in a nonhomogeneous pattern throughout the nucleoplasm, excluding nucleoli. They are most concentrated in coiled bodies, but in addition are present in “speckled” structures which are distinct from coiled bodies and which contain the non-snRNP splicing factor SC-35. U1 snRNP shows a more widespread nucleoplasmic staining, outside of coiled bodies and “speckled” structures, relative to the other snRNPs. The association of snRNPs with “speckles” is disrupted by heat shock but enhanced when cells are treated with α -amanitin.

THE development of in vitro splicing extracts from mammalian and yeast systems has resulted in major advances in understanding the basic mechanism of pre-mRNA splicing (for reviews see references 9, 27, 38, 41, 54). The splicing reaction involves two sequential transesterification reactions, similar to that seen with group II self-splicing introns (reviewed in reference 31). Unlike self-splicing introns however, nuclear pre-mRNA splicing requires a complex set of trans-acting splicing factors which bind to substrate RNAs in an ordered pathway to form an active splicing complex or “spliceosome” (8, 17, 22, 26, 32, and 47). Major components of spliceosomes are the U1, U2, U4/U6, and U5 snRNPs, all of which are required for both spliceosome assembly and splicing. In addition to these snRNPs, recent studies have identified a number of non-snRNP protein factors which are also required for splicing in vitro (4, 23, 25, 33, 34, 55, 69, 74, 75).

In contrast with the substantial progress in dissecting the splicing machinery in vitro, comparatively little is known about how the splicing factors are organized in vivo, or how splicing is integrated in the nucleus with transcription or RNA processing reactions such as 3' polyadenylation. Many previous studies have shown by indirect immunofluorescence that autoimmune and monoclonal antibodies specific for

snRNP proteins, or for the snRNA-specific 2,2,7 trimethyl guanosine cap structure (m3G-Cap),¹ stain the nucleoplasm of mammalian cells in a characteristic pattern which is often described as “speckled” or “punctate” (20, 28, 44, 45, 52, 58, 59, 60, 70). A mAb specific for a non-snRNP splicing factor (SC-35), was shown to label a similar pattern of 20–50 “speckled” nuclear structures (23). In contrast, an anti-peptide antibody raised against a different non-snRNP splicing factor (U2AF), showed widespread nucleoplasmic labeling, rather than “speckles,” and also stained a small number of discrete, snRNP-containing foci (15, 75). Antibodies against hnRNP proteins also stain throughout the nucleoplasm, excluding nucleoli, without obvious accumulation in either “speckles” or foci (18, 48).

Recent studies on amphibian oocytes reveal a different pattern of snRNP labeling (reviewed in reference 24). Antibodies specific for snRNP proteins and m3G-Cap structures stain loops on lampbrush chromosomes and at least three classes of extrachromosomal granules, termed A, B, and C “snurposomes.” A snurposomes contain only U1 snRNP while B snurposomes contain all the spliceosomal snRNPs,

1. Abbreviation used in this paper: m3G-Cap, trimethyl guanosine cap.

in approximately stoichiometric amounts, as determined by in situ hybridization with ³H-labeled probes. B snurposomes and lampbrush chromosome loops also label strongly with the anti-SC-35 mAb (72). C snurposomes vary considerably in size, but can be as large as 20 μm and usually have several B snurposomes on their surface. The large C snurposomes have previously been described as "sphere organelles" (14). The relationship between snRNP-containing structures in the nuclei of mammalian cells and the snurposomes in amphibian oocytes is at present unclear.

Most previous studies on snRNP localization in mammalian cells have been based largely or exclusively on the use of antibodies specific for either common snRNP proteins (Sm class) or the m3G-Cap structure which is common to the U class of snRNAs, with the exception of U6 (reviewed in 41 and 76). These probes are valuable for identifying sites where snRNPs are present in situ, but do not distinguish which species of snRNPs are present at any particular site. As the individual snRNPs involved in splicing bind to pre-mRNAs in a stepwise pathway during spliceosome formation in vitro, it is important to determine whether this is reflected by any differences in their nuclear localization in vivo. This requires probes which can specifically label each individual snRNP species. We have recently addressed these questions by using antisense oligonucleotide probes specific for individual snRNAs (15, 16). These antisense probes are made of 2'-*o*-alkyl RNA and have been shown to bind highly specifically to targeted snRNAs in vitro (5, 6, 10, 36, and 37). This approach also affords the possibility of establishing that fully assembled snRNPs are being detected, by double labeling with antisense probes and antibodies specific for snRNP proteins.

Our previous studies using the antisense probes showed that U2, U4, U5, and U6 snRNAs were strongly labeled in discrete nucleoplasmic foci, while U1 snRNA, in addition to being in the foci, was widely distributed throughout the nucleoplasm, excluding nucleoli. An antibody specific for the splicing factor U2AF showed a similar nucleoplasmic staining to U1 snRNP and also concentrated in the same foci (15, 75). By microinjecting 2'-*o*-alkyl probes coupled to fluorochromes into the nuclei of unfixed, living cells it was also possible to demonstrate a similar pattern of snRNP labeling in vivo (16).

In this study we extend our analysis of the snRNP-rich nuclear foci and show that they are distinct from other nucleoplasmic structures which form the overall "speckled pattern" in cells stained with snRNP-specific antibodies. In particular, we identify the foci as being coiled bodies and present evidence that the association of snRNPs with foci is linked to nuclear pre-mRNA metabolism.

Materials and Methods

Oligonucleotide Synthesis and Labeling

Oligonucleotides were synthesized as described by Sproat et al. (63). All oligonucleotides were made of either 2'-*o*-methyl (63) or 2'-*o*-allyl RNA (30) as indicated in the legend to Table I. Sequences of the oligonucleotides are listed in Table I. Biotinylation of antisense probes was performed during the solid phase synthesis as previously described (46).

Cell Culture

HeLa cells were grown on glass coverslips in MEM supplemented with 1%

Table I. Sequence of Oligonucleotides

Oligonucleotide	Catalogue number	Sequence (5'-3')
anti-U1	8	CCUICCAIUAAIUAAU
anti-U2	516	IAACAIAUACUACACUU
anti-U3	543	UUUCIUIUCUC
anti-U4	112	CUGCCACUGCGCAAAGCU
anti-U5	194	UA*GUA*A*A*GCGC
anti-U6	191	A*UGGA*A*CGCUUCA*CGA*A*UUU
anti-28Sa	525	AIAICCAAUCCUUAAU
anti-28Sb	523	IUCCIUUUUCAAIA
Ad1c	147	IACCAIAUIIACICICIC

All oligonucleotides were made of 2'-*o*-allyl RNA (30), except for reference numbers 8, 112, and 147, which were 2'-*o*-methyl RNA. The catalogue number refers to the laboratory catalogue identification number for each probe. The oligonucleotides were either 5' or 3' end labeled with biotin (63). The bases marked A* correspond to 2-aminoadenosine (36, 65, and 65). Other bases used were uridine (U), adenosine (A), inosine (I), guanosine (G), and cytosine (C).

glutamine, 10% FCS, and antibiotics (Gibco BRL, Grand Island, NY). Cells were used after reaching a confluency of 50-80%.

For drug treatment cells on coverslips were placed in fresh medium containing either 5 μg/ml actinomycin D (Sigma Chemical Co., St. Louis, MO) or 50 μg/ml α-amanitin (Sigma Chemical Co.). Microinjection of α-amanitin was performed using the AIS microinjection system previously described (2, 3).

For heat shock experiments the coverslips were transferred to Petri dishes or flasks containing pre-warmed (45°C) medium supplemented with 10 mM Hepes, pH 7.4, and incubated in a 45°C water bath for 15 min. Control cells were also transferred to dishes and incubated for the same time in medium kept at 37°C.

In Situ Hybridization and Immunofluorescence

Cells on coverslips were washed twice with PBS and processed according to either of the following protocols: (a) cells were extracted with 0.5% Triton X-100 in CSK buffer for 30 s on ice and fixed in 3.7% paraformaldehyde in the same buffer for 10 min at room temperature (15); or (b) cells were fixed with 3.7% paraformaldehyde in CSK buffer for 10 min, washed in PBS (3 × 5 min), and subsequently extracted with 0.2% SDS (molecular biology grade, Sigma Chemical Co.) 20 mM Tris, pH 7.4, 30 mM NaCl, and 2.5 mM EDTA, for 15 min at room temperature, with gentle shaking. After extensive washing in PBS the cells were hybridized with biotinylated antisense 2'-*o*-alkyl RNA probes (final concentration 1 pM/μl) in a humid chamber for 1 h at room temperature, or overnight at 4°C, as previously described (15). Hybridization sites were detected with FITC-conjugated extravidin (Sigma Chemical Co.) diluted 1:500 in avidin buffer (15). Immunofluorescence staining was performed as previously described (15), except that Tween 20 was frequently omitted from the PBS washes. Double labeling with antisense and antibody probes was performed as previously described (15). For double immunolabeling the cells were consecutively incubated with one primary antibody, appropriate fluorochrome-conjugated secondary antibody, then the other primary antibody and fluorochrome conjugate. Note that double labeling with the 3C5 monoclonal and the other mAbs used (see below for list of other antibodies) was possible because 3C5 is IgM and all others are IgG. Specificity of double labeling using monoclonal 3C5 (IgM) and other mAbs (IgGs) was controlled by reacting cells with either primary antibody alone, followed by incubation with either anti-IgM or anti-IgG conjugates.

The following antibodies were used: mAbs anti-70K protein (7), anti-B⁺ protein ("4G3") (28), anti-2,2,7-trimethylguanosine cap structures (11), anti-SC-35 splicing factor (23), anti-hnRNP peptides C1-C2 ("4F4") and L ("4DI") (18,48), and monoclonal "3C5" (68); p80 coilin-specific human antiserum "Sh" (1) and rabbit antibodies raised against a β-galactosidase fusion protein containing the COOH-terminal region of p80 coilin (1).

Preparations were examined in a fluorescence microscope (IM35; Carl Zeiss, Oberkochen, Germany) using a 100× objective. Images were recorded using a Hamamatsu SIT-camera with a DVS3000 system (Hamamatsu Photonics, Japan). Confocal fluorescence microscopy was performed using the EMBL modular confocal microscope as previously described (15).

Results

In Situ Labeling of snRNPs

In our previous studies it was demonstrated for both U1 and U3 snRNPs that the in situ staining patterns of antisense and antibody probes colocalized (15). However, with the same Triton X-100 pre-extraction conditions used in these studies, a mAb which recognizes the U2 snRNP-specific protein B^{''} labeled not only bright foci, but also additional nucleoplasmic structures not detected by the U2 antisense probe (Fig. 1, compare *A* and *B*). As previously discussed, the failure to detect perfect colocalization of antibody and antisense probes for U2 snRNP could have been due either to the presence of free B^{''} protein in the nucleus (i.e., not assembled with U2 snRNA), or to the presence of U2 snRNP in structures where the binding site for the antisense probe was not accessible. To try to resolve this question we have systematically examined different in situ labeling procedures with the aim of uncovering specific labeling of U2 snRNA in the additional structures stained by the anti-B^{''} antibody.

Using a novel procedure, involving SDS extraction of formaldehyde-fixed cells (see Materials and Methods), we now observe colocalization of the labeling patterns of the U2-specific antisense and antibody probes (Fig. 1, compare *C* and *D*; see also Fig. 3, *B* and *E*). With both probes, the foci are stained more brightly than any other nuclear structure, confirming that they are the most concentrated sites of U2 snRNP accumulation (Fig. 1, *arrows*). Both probes also reveal U2 snRNP distributed throughout the nucleoplasm in a non-homogeneous pattern. This pattern includes additional concentrated staining regions giving the appearance of

“speckles” (Fig. 1, *arrowheads*). Having now obtained conditions whereby antibody and antisense probes to U2 snRNP co-localize, we next sought to carefully control whether this SDS extraction method produced specific labeling of U2 and other RNA species (Figs. 2 and 3).

Nine antisense probes complementary to different RNA targets were tested (Fig. 2). The U1-specific probe (Fig. 2 *A*) showed the same widespread nucleoplasmic distribution observed previously (15, 16). As with previous methods, the U1 antisense probe colocalized with the staining pattern of a mAb which recognizes the U1-specific 70K antigen (data not shown). The U1 staining pattern excludes nucleoli, but includes staining of foci and “speckles,” together with considerable additional nucleoplasmic labeling. In contrast, each of the U2, U4, U5, and U6 snRNA-specific probes showed a similar labeling pattern to each other (Fig. 2, *B–E*). In each case we observe bright staining in foci plus additional nucleoplasmic labeling, excluding nucleoli. Within the nucleoplasmic staining pattern each probe showed more concentrated labeling of “speckled” structures. As a negative control, a probe not complementary to any RNA known to be expressed in HeLa cells was tested and showed no specific labeling of either nuclear or cytoplasmic structures (Fig. 2 *F*). A probe for the nucleolar snRNA, U3, exclusively labeled nucleoli (Fig. 2 *G*). Two probes specific for separate regions of 28S rRNA both strongly labeled the cytoplasm and nucleoli, as expected, but not the nucleoplasm (Fig. 2, *H* and *I*).

Competitive hybridization experiments were also performed to examine the specificity of the different labeling patterns obtained using the SDS method (Fig. 3). Each of the U1, U2, and U3 snRNA probes was hybridized in situ to-

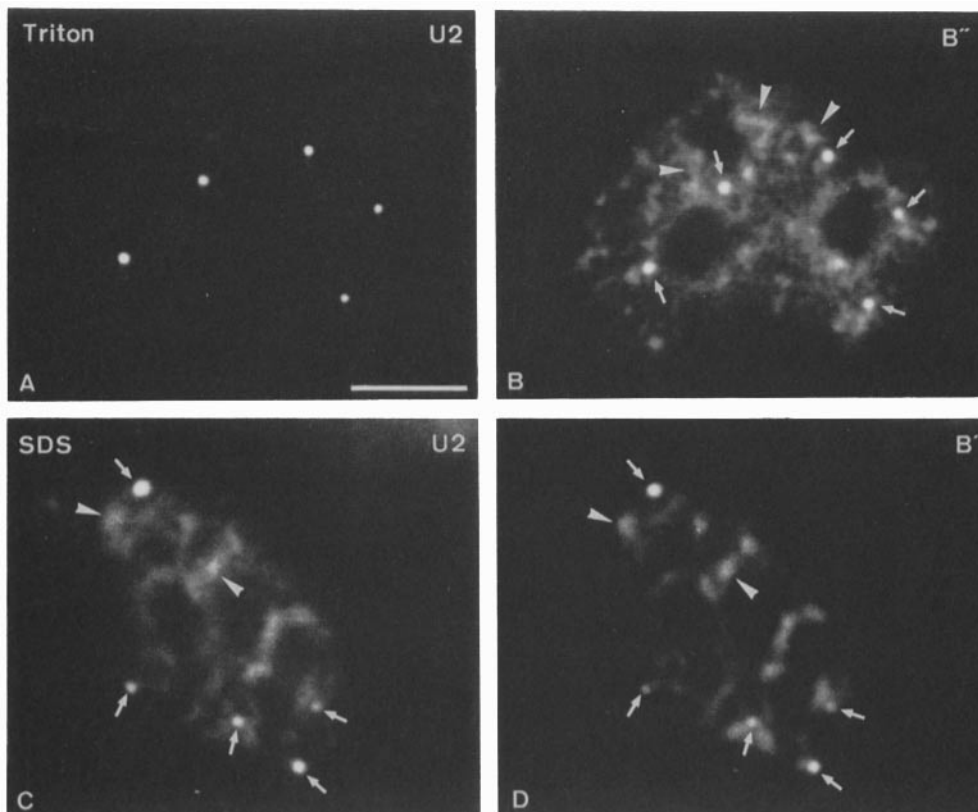


Figure 1. In situ labeling of U2snRNP. HeLa cells were extracted with Triton X-100 (*A* and *B*) or SDS (*C* and *D*). On Triton-extracted cells the U2 antisense probe labels exclusively foci (*A*). Double labeling with a U2-specific mAb (anti-B^{''}) reveals the foci (*arrows*) and additional nucleoplasmic staining (*arrowheads*) (*B*). After SDS extraction both antisense (*C*) and antibody (*D*) probes colocalize and label foci (*arrows*) and nucleoplasmic speckled structures (*arrowheads*). Bar, 10 μ m.

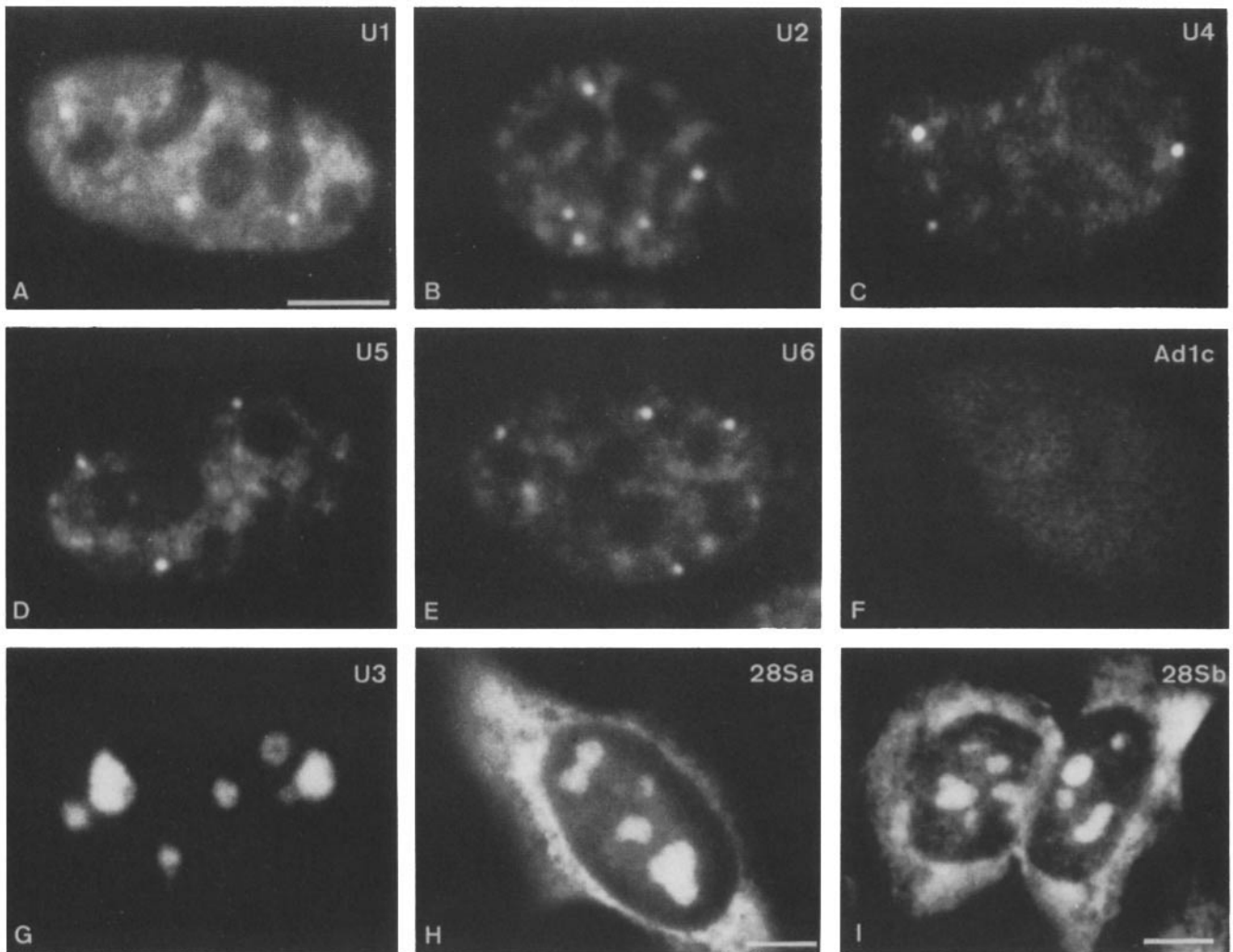


Figure 2. In situ hybridization on SDS-extracted cells. HeLa cells were incubated with antisense probes complementary to U1 (A), U2 (B), U4 (C), U5 (D), and U6 (E) snRNAs; the first intron of the adenovirus major late transcript (F); U3 snRNA (G), and 28S rRNA (H and I). Bar, 10 μ m.

gether with a large excess of nonbiotinylated probe complementary to a different snRNA species (Fig. 3, A–C). In each case, the antisense labeling pattern was not competed by the non-cognate competitor. Parallel double-labeling with the U2-specific anti-B^{''} antibody also demonstrated that only the U2 antisense probe colocalized (Fig. 3, compare B and E with A and D and C and F). When the antisense probes were hybridized together with a large excess of nonbiotinylated probe of identical sequence however nuclear staining was lost in each case (Fig. 3, G–I). As an internal control, double labeling was again performed with the anti-B^{''} antibody and shown to be unaffected (Fig. 3, J–L).

The fact that antisense probes specific for U3 or rRNA label only nucleoli, or nucleoli and cytoplasm, but not nucleoplasmic structures, while probes for each of the snRNAs known to be required for pre-mRNA splicing exclusively label the nucleoplasm, demonstrates that the general localization pattern of RNA complexes is not perturbed by the SDS method. This is further supported by the labeling patterns obtained with antibodies specific for hnRNP pro-

teins, snRNP proteins, and splicing factors using the SDS method, all of which are similar to the labeling patterns seen previously with these reagents using alternative in situ fixation and staining procedures (this work and other data not shown). We conclude that the SDS method described here allows efficient and highly specific in situ detection of RNA-protein complexes in mammalian cells.

snRNP-rich Foci Are Distinct from Other snRNP-containing Nucleoplasmic "Speckles"

Having established that each of the U1, U2, U4/U6, and U5 snRNPs are present in all the nuclear structures that make up the overall "speckled" labeling pattern (Figs. 1–3), we next addressed whether the strongly staining foci were the same as, or distinct from, the other nucleoplasmic "speckles." In previous EM studies it was observed that nuclear structures called interchromatin granules were strongly labeled by anti-Sm antibodies (21, 61). Based on these observations it was proposed that clusters of inter-

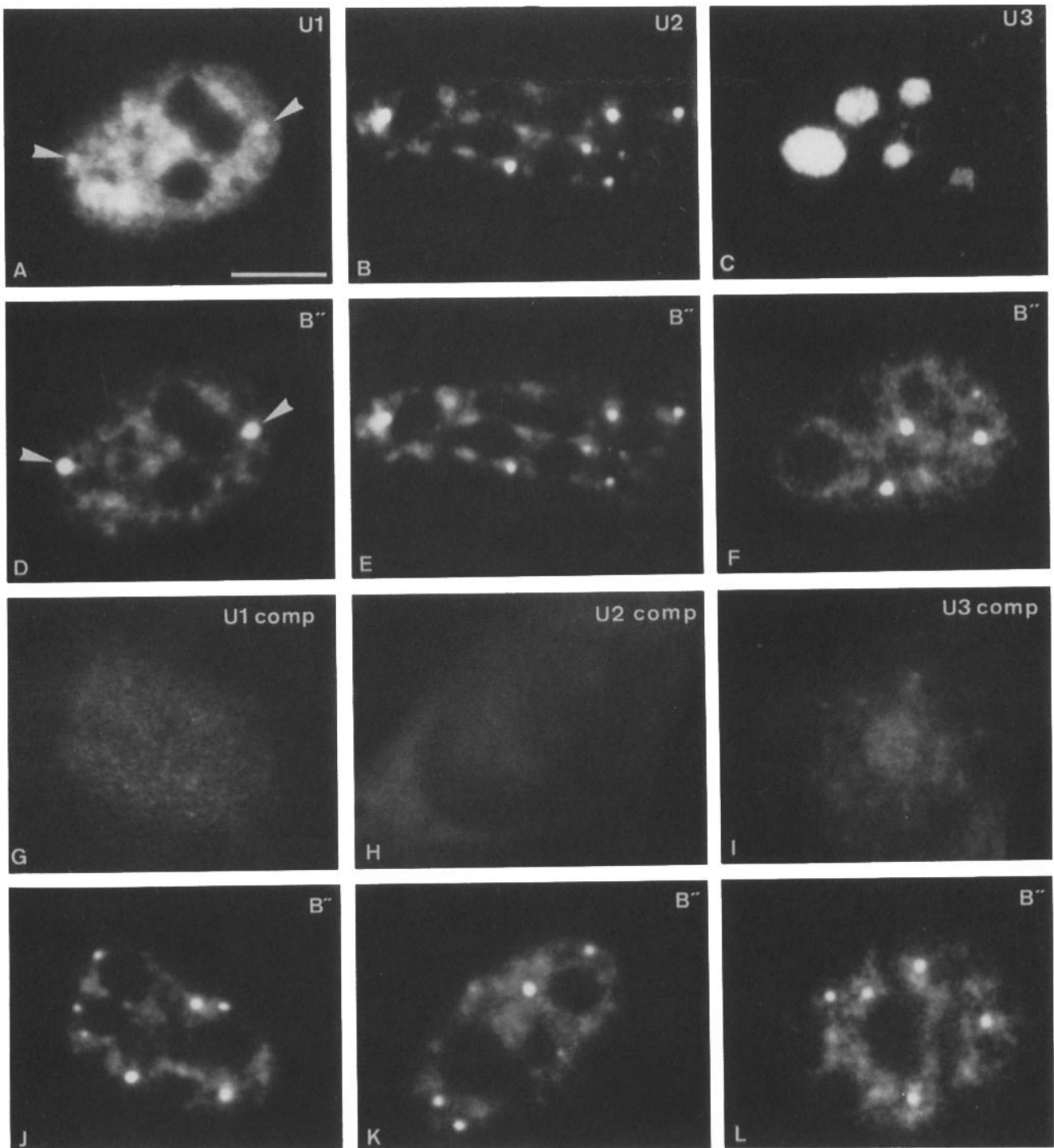


Figure 3. Competitive hybridization experiments on SDS-extracted cells. Each of the U1 (A), U2 (B), and U3 (C) snRNA probes was hybridized in situ together with a 100-fold molar excess of non-biotinylated probe of different sequence, complementary to, respectively, U3 (A and B) or U2 (C) snRNAs. Cells were double labeled with the U2-specific anti-B'' antibody (D, E, and F). In situ hybridization with U1 (G), U2 (H), and U3 (I) snRNA probes in the presence of 100-fold molar excess of nonbiotinylated probe of identical sequence abolishes nuclear staining but does not interfere with anti-B'' antibody labeling (J, K, L). Bar, 10 μ m.

chromatin granules are the "speckled" structures seen in the light microscope. A mAb (3C5) has been described which predominantly stains interchromatin granules (67, 68). Interestingly, the 3C5 monoclonal has been shown to recognize a group of proteins on Western blots through a shared phos-

phorylated epitope (66). We therefore used this monoclonal to compare its in situ labeling pattern with the U2 snRNP-specific anti-B'' antibody (Fig. 4, A-C).

Double labeling with 3C5 and anti-B'' shows that the foci, which are brightly stained by the anti-B'' antibody (Fig. 4 A,

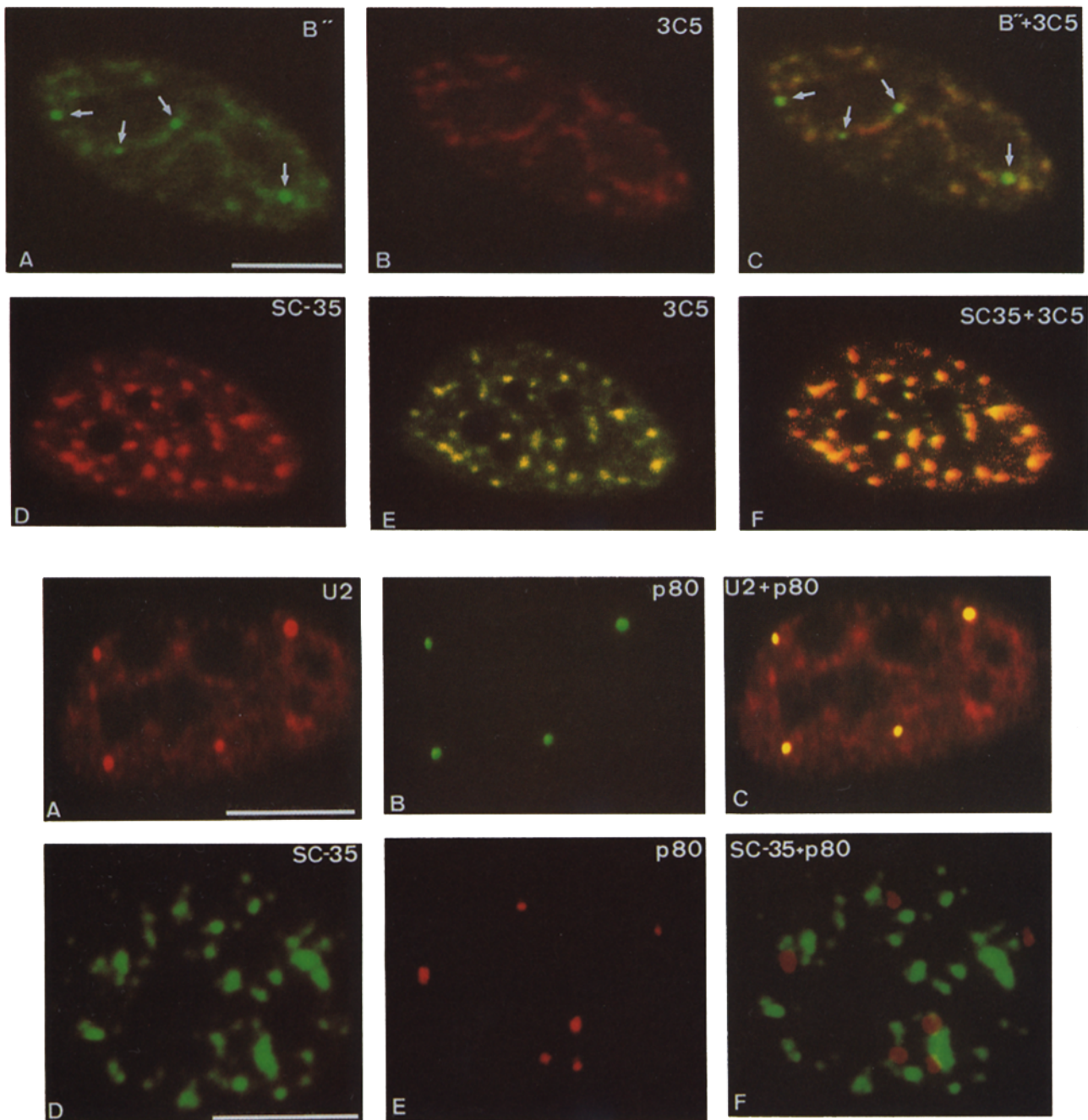


Figure 4. snRNP-rich foci are distinct from “speckles.” HeLa cells extracted with Triton X-100 were double labeled with the U2-specific anti-B’ (A) and 3C5 (B) antibodies. The overlay (C) shows that the foci stained by the anti-B’ antibody (arrows) are not labeled by the 3C5 monoclonal, although the speckled structures do colocalize. Double labeling with SC35 (D) and 3C5 (E) mAbs shows that they colocalize in “speckles” (F). In the overlays (C–F) colocalization of labeling results in a change in color. Bar, 10 μ m.

Figure 5. snRNP-rich foci are coiled bodies. HeLa cells extracted with SDS were double labeled with the U2 snRNA probe (A) and anti-p80 coilin antibody (B). The overlay shows that both probes label the same foci (C). Double labeling on SDS-extracted cells with SC-35 (D) and anti-p80 coilin (E) antibodies reveals no colocalization in the overlay (F). Bar, 10 μ m.

arrows), do not colocalize with the 3C5 monoclonal (Fig. 4 C, arrows; note green foci). However, the additional “speckled” structures labeled by the anti-B’ antibody do colocalize with the 3C5 monoclonal (Fig. 4 C, orange “speckles”). This indicates that only these additional “speckles,” and not the foci, correspond to interchromatin granules. Another mAb,

called SC-35 (23), which recognizes a non-snRNP splicing factor, also stains the speckled structures but not the bright foci (16). A double-labeling experiment with mAbs SC-35 and 3C5 shows that their staining patterns colocalize in “speckles” (Fig. 4, D–F), indicating that the anti-SC-35 antibody is also staining interchromatin granules. This agrees

with the recent data of Spector et al. (62) which show at the EM level that interchromatin granules and perichromatin fibrils are stained by the anti-SC-35 antibody.

We conclude from these data: (a) the brightly staining foci are distinct structures in the overall "speckled" pattern; (b) the foci differ from the interchromatin granules stained by the 3C5 and anti-SC-35 mAbs; and (c) in addition to being in foci, U1, U2, U4/U6, and U5 snRNPs are each also present in interchromatin granules.

The snRNP-rich Foci Correspond to Coiled Bodies

In addition to interchromatin granules, Fakan et al. (21), reported that at the EM level perichromatin fibrils and coiled bodies were also nuclear structures preferentially stained by anti-Sm antibodies. Recently, a new class of autoimmune antibodies has been characterized which specifically label coiled bodies (50, 51). These antibodies recognize a protein of 80 kD, termed p80 coilin. Antibodies affinity purified using the COOH-terminal fragment of p80 coilin, expressed from a cDNA clone, also specifically label coiled bodies (1). Coiled bodies are also stained specifically by rabbit antibodies raised against recombinant p80 coilin. We have therefore used both coilin-specific human sera and antibodies raised against recombinant p80 coilin to determine whether the snRNP-rich foci correspond to coiled bodies (Fig. 5).

A perfect colocalization was observed between p80 coilin staining and the foci detected using the U2-specific antisense probe (Fig. 5, A-C, yellow structures in C). All oligonucleotide and antibody probes which label the foci precisely colocalize with anti-p80 coilin staining in double-labeling experiments (this work and other data not shown). As an additional control, double labeling was done with anti-SC-35 and anti-p80 coilin antibodies (Fig. 5, D-F). As expected from our previous studies (16), no overlap or colocalization was observed.

We conclude that the snRNP-rich nuclear foci correspond to coiled bodies. These data also indicate, in agreement with the findings of Raska et al. (51), that anti-p80 coilin antibodies do not label interchromatin granules and do not colocalize with the SC-35 antigen.

Association of snRNPs with Coiled Bodies Is Disrupted by Heat Shock

The heat shock response is known to cause a major change in transcriptional activity *in vivo*, resulting in most RNA polymerase II transcripts being inhibited with a concomitant induction of specific "heat shock" genes (for reviews see references 19, 39). There is also evidence that pre-mRNA splicing can be inhibited by heat shock both *in vivo* and *in vitro* (12, 13, and 73). It has also been shown that heat shock affects snRNP-snRNP interactions and in particular disrupts the U4/U5/U6 triple snRNP (12). We have therefore analyzed how the *in situ* distribution of each snRNP species is affected during heat shock and subsequent recovery (Fig. 6).

HeLa cells were heat shocked by a temperature shift from 37° to 45°C for 15 min. In the majority of cells a dramatic change was observed in the nuclear distribution of snRNPs. Both antibody and antisense probes show a widespread, rather diffuse nucleoplasmic snRNP staining after heat shock, although nucleolar exclusion is maintained (Fig. 6, A-C, and other data not shown). We note that colocalization of antibody and antisense probes is still observed after heat

shock, indicating that the protein and snRNA components have not been dissociated. For both U1 and U2 snRNPs, double labeling with antisense probes and the anti-p80 coilin antibody also shows that they no longer concentrate in coiled bodies (Fig. 6, compare A and D and B and E). Similar results are obtained with probes for the other splicing snRNPs (data not shown). Foci of p80 coilin staining are still observed after heat shock (Fig. 6, D and E), although some cells show a partial disruption of the usual regular appearance of coiled bodies seen in non-heat-shocked cells. In agreement with the recent findings of Spector et al. (62), we observe that the association of snRNPs with "speckled" structures is also disrupted by heat shock. However, U3 snRNP is still detected exclusively in nucleoli after a similar heat shock (Fig. 6 F).

In cells maintained at 37°C, or allowed to recover at 37°C for 15 min after heat shock, snRNPs are again observed to concentrate in coiled bodies (Fig. 6, G-I and J-L). Note that after recovery from heat shock the staining pattern is restored to that seen in non-heat shocked cells, including the typical bright labeling of foci and additional nucleoplasmic "speckles" (Fig. 6 I).

We conclude: (a) heat shock affects the association of each of the spliceosomal snRNPs with coiled bodies and (b) the spliceosomal snRNPs are apparently no longer localized to specific nucleoplasmic structures during the heat shock response but nevertheless remain in the nucleoplasm and do not enter the cytoplasm or nucleoli.

snRNPs Do Not Colocalize with p80 Coilin when Transcription Is Inhibited by Actinomycin D

If the presence of snRNPs in coiled bodies is connected with the processing or metabolism of pre-mRNA then this association might be dependent upon the production of nascent transcripts in the nucleus. Experiments were therefore done to assess how the nuclear snRNP distribution is affected by blocking transcription with either actinomycin D or α -amanitin (see Figs. 7-10).

We have previously shown that treating cells with actinomycin D, which inhibits all nuclear transcription, results in an astonishing change in the U1 snRNP labeling pattern (15, 16). In actinomycin-treated cells both U1 snRNP and the splicing factor U2AF, but not the other spliceosomal snRNPs, hnRNP proteins or the splicing factor SC-35, become concentrated around remnants of nucleoli. In contrast, the U2, U4/U6, and U5 snRNPs, and the splicing factor SC-35, remain colocalized in "speckled" nucleoplasmic structures (16; and other data not shown). These earlier studies could not however distinguish whether any of these "speckled" staining structures observed after actinomycin D treatment correspond to foci/coiled bodies.

Double-labeling experiments were carried out with both an antisense probe and mAb specific for U2 snRNP and the anti-p80 coilin antibody, before and after actinomycin D treatment (Fig. 7, compare A and D and B and E). This shows that U2 snRNP no longer colocalizes with p80 coilin after cells are exposed to actinomycin D. Similar results are obtained with probes specific for U4/U6 or U5 snRNPs (data not shown). Each of these snRNPs still show concentrated staining in "speckled" structures however and do not appear widely dispersed in the nucleoplasm as during the heat-shock response. The anti-p80 coilin staining is also

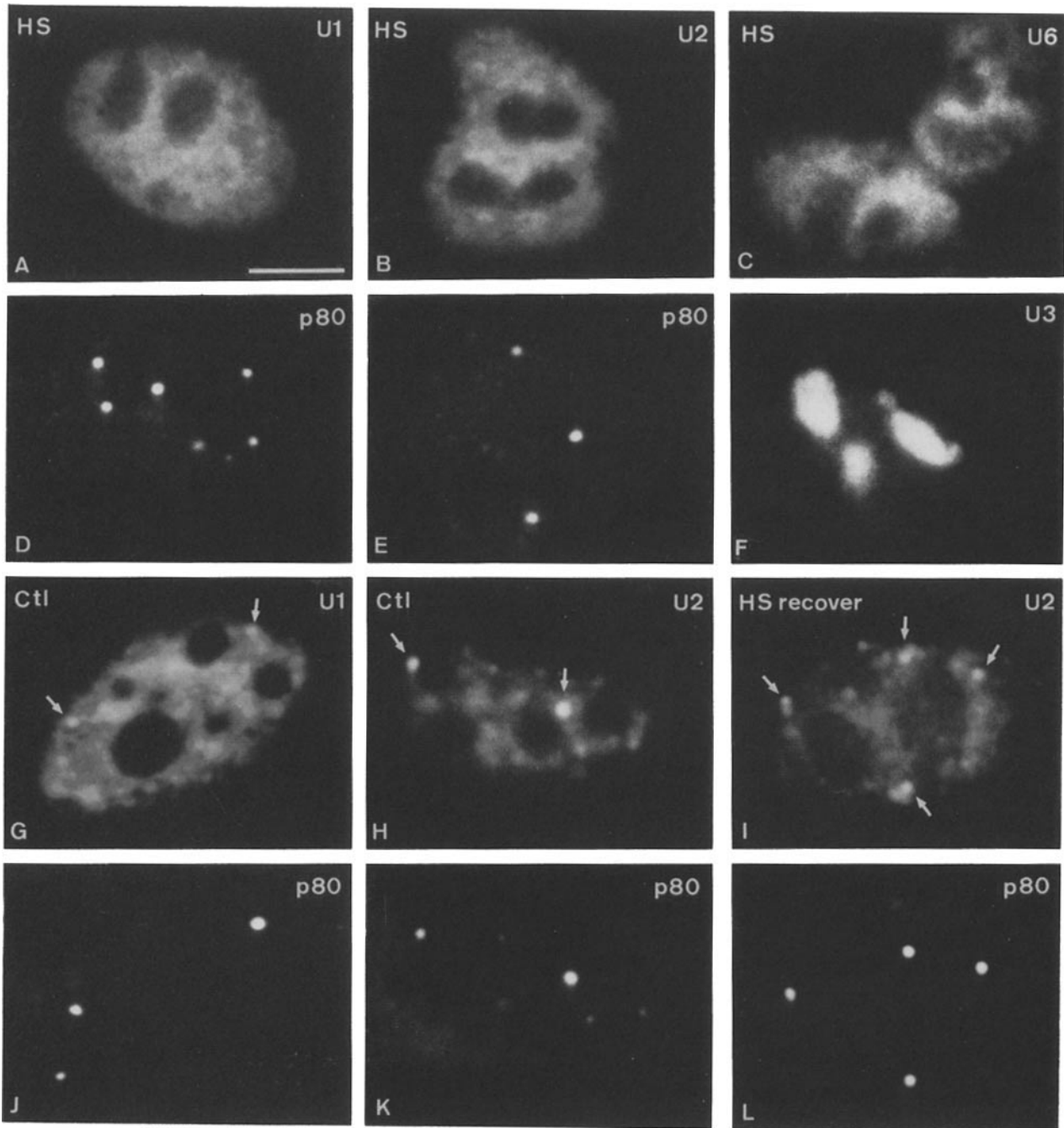


Figure 6. Heat shock affects the distribution of snRNPs. HeLa cells were heat-shocked at 45°C for 15 min before fixation and SDS extraction (A–F). Antisense probes to U1 (A), U2 (B), and U6 (C) snRNAs show a widespread uniform labeling of the nucleoplasm, excluding nucleoli. The pattern of p80 coilin staining in the same cells remains unaffected (D and E). The U3 snRNA in heat-shocked cells is still detected exclusively in the nucleolus (F). Control cells incubated at 37°C and double labeled with U1 (G) or U2 (H) snRNA probes and anti-p80 coilin antibody (J and K) show the characteristic snRNP-rich foci (arrows). Note that foci labeled with the U1snRNA probe appear less prominent due to the intense widespread staining of the nucleoplasm (G). When cells are allowed to recover at 37°C for 15 min after heat shock, the U2snRNA (I, arrows) is again concentrated in foci which colocalize with p80 coilin (L). Bar, 10 μ m.

changed after actinomycin D treatment (50), and appears clustered around nucleolar remnants in a distinct, though possibly overlapping, pattern to that seen for U1 snRNP and U2AF (Fig. 7, compare C and F; see also reference 16).

We conclude that spliceosomal snRNPs no longer concentrate in coiled bodies after actinomycin D treatment. Al-

though we had previously observed nucleoplasmic “foci,” containing U2, U4/U6, and U5 snRNPs, which remained after actinomycin D treatment (15), it is now clear from the double labeling with anti-p80 coilin antibodies that these structures are different from the brightly staining foci (coiled bodies) seen in untreated cells. These snRNP-containing

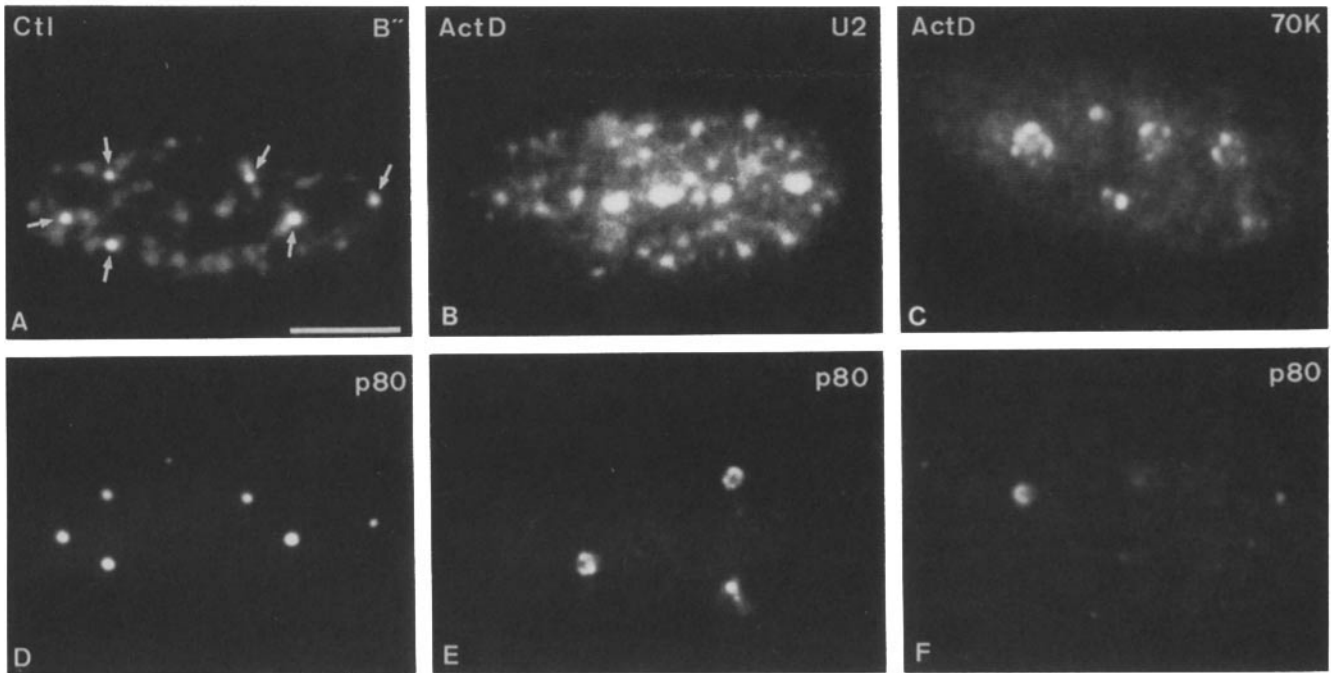


Figure 7. Actinomycin D disrupts coiled bodies. HeLa cells extracted with Triton X-100 and labeled with U2-specific anti-B' antibody (A) show foci (arrows) which perfectly colocalize with anti-p80 coilin (B). After 1-h treatment with actinomycin D (5 $\mu\text{g}/\text{ml}$) the U2 antisense probe (B) shows an altered staining pattern which no longer colocalize with p80 coilin (E). Double labeling of actinomycin D-treated cells with the U1-specific anti-70K mAb (C) and anti-p80 coilin antibody (F) shows that p80 coilin clusters around the nucleolus in a nonidentical pattern to that seen for U1snRNP. Bar, 10 μm .

nucleoplasmic "speckled" structures also label with the anti-SC-35 and 3C5 mAbs and therefore likely correspond to, or are derived from, the "speckles" present in untreated cells.

snRNPs Concentrate in "Speckles" but not Coiled Bodies after α -Amanitin Treatment

Actinomycin D inhibits rRNA as well as mRNA and tRNA synthesis and causes significant changes in nuclear morphology, including nucleolar breakdown. In contrast, α -amanitin selectively inhibits transcription by RNA polymerase II and, at higher concentrations, RNA polymerase III, but does not block rRNA synthesis. We therefore tested whether treatment of HeLa cells with α -amanitin also affected the in situ distribution of snRNPs between the different nuclear compartments (see Figs. 8–10).

HeLa cells were incubated for 5 h in culture medium containing α -amanitin as previously described (40). A major change in the pattern of U2 snRNP staining was observed (Fig. 8, compare A and B). Both the U2-specific antisense probe and mAb (anti-B') intensely label nucleoplasmic "speckles" after α -amanitin treatment (Fig. 8, B, C, and F). Note that a perfect colocalization of the U2 antisense and antibody probes is still seen after α -amanitin treatment (Fig. 8, C and F). However, neither probe now shows U2 colocalized with p80 coilin (Fig. 8, compare A and D with B and E, and data not shown). p80 coilin staining was also affected by α -amanitin; in most cases foci were disrupted and many cells showed "caps" of p80 coilin clustered around nucleoli (Fig. 8, compare D and E).

After α -amanitin treatment the mAbs anti-B' and 3C5

colocalize in the large, bright "speckles" (Fig. 8, compare G and J with H and K). A monoclonal anti-m3G-Cap antibody also colocalizes in the large "speckles" with 3C5 on α -amanitin-treated cells (Fig. 8, compare I and L), as does SC-35 (data not shown). The same structures are labeled with the 3C5 antibody and probes specific for U1 (Fig. 9, compare A and D) and U4/U6 and U5 snRNAs (data not shown). Unlike the other spliceosomal snRNPs, U1 is additionally detected in perinucleolar structures after α -amanitin treatment (Fig. 9 A). These data again demonstrate a difference between U1 and the other spliceosomal snRNPs. α -amanitin does not produce any major changes in the pattern of hnRNP staining and does not cause hnRNPs to colocalize in the "speckles" stained by the 3C5 mAb (Fig. 9, compare B and E and C and F). Note that concentrated regions of hnRNP L persist after α -amanitin treatment, but do not obviously colocalize with any of the structures labeled by 3C5 (Fig. 9, compare C and F).

The effect of α -amanitin has also been studied by directly injecting it into the nuclei of HeLa cells and then comparing the snRNP distribution in both injected and uninjected cells. In all injected cells, the anti-U2 staining was concentrated in large, bright, nucleoplasmic "speckles" (Fig. 10 A). These "speckles" colocalized with the 3C5 monoclonal (data not shown). Again, after α -amanitin, colocalization was no longer observed between anti-U2 snRNP and anti-p80 coilin staining (Fig. 10, compare A and B). In a few cells, foci of p80 coilin staining could still be seen, but snRNPs were not concentrated in these structures (Fig. 10, arrows). Interestingly, these residual foci were often observed adjacent to enlarged speckles (Fig. 10 A, arrows and arrowheads). An es-

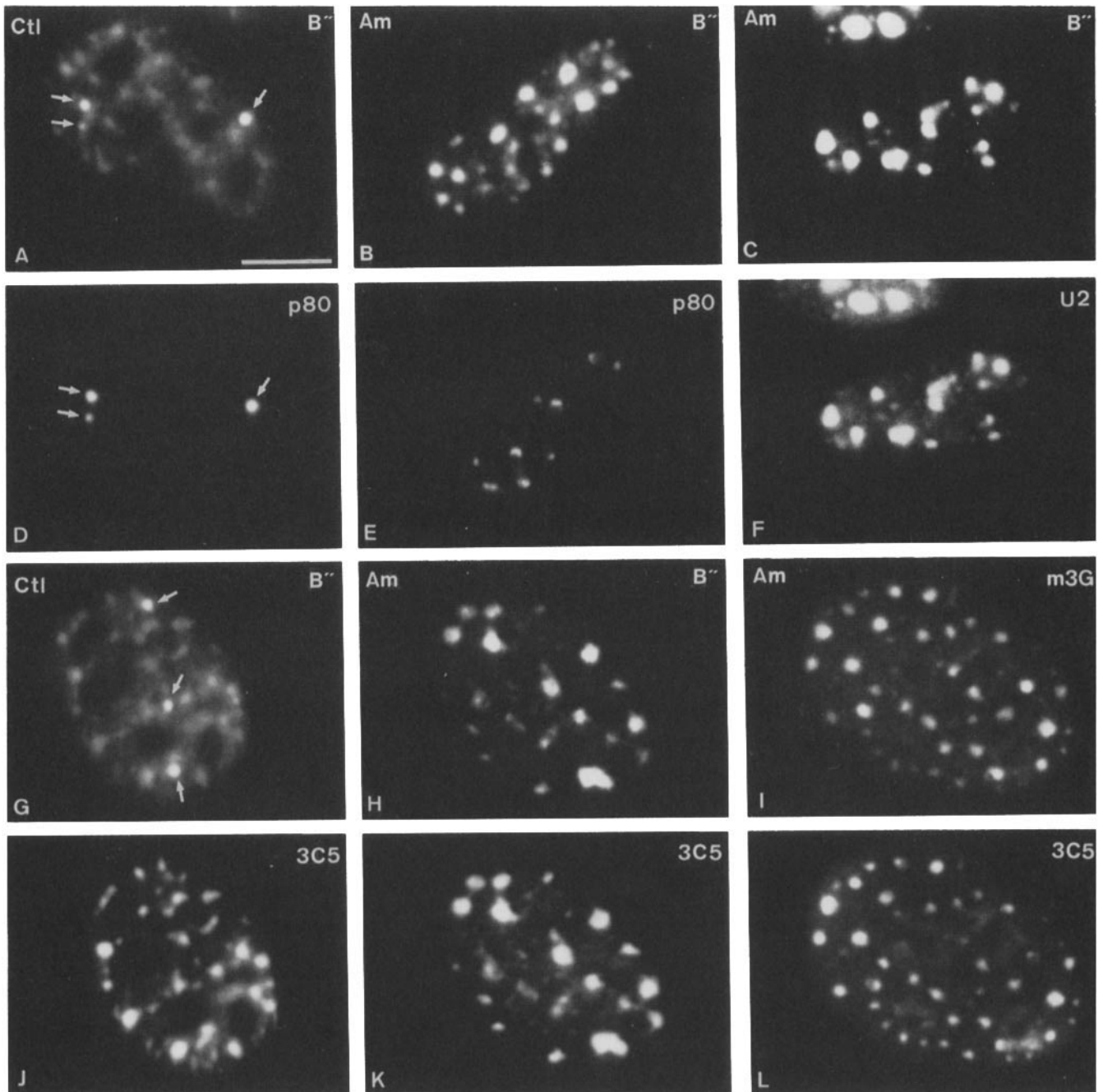


Figure 8. snRNPs concentrate in "speckles" after amanitin treatment. In control experiments HeLa cells were extracted with Triton X-100 and double labeled with the U2-specific anti-B' (A) and anti-p80 coilin (D) antibodies. U2snRNP is concentrated in coiled bodies (arrows) and additionally is present in nucleoplasmic "speckles." After 5-h exposure to α -amanitin (50 μ g/ml) the anti-B' staining concentrates in large, bright speckles (B) which are distinct from the cap-like structures associated with the nucleolus seen with the anti-p80 coilin antibody (E). Double labeling of α -amanitin-treated cells with anti-B' antibody (C) and U2snRNA antisense probe (F) show perfect colocalization. In control, Triton-extracted cells, double labeling with anti-B' (G) and 3C5 (J) antibodies shows that only the "speckles" and not the bright foci (arrows) colocalize. After amanitin treatment there is a perfect colocalization of anti-B' (H) and 3C5 (K) staining. Colocalization is also observed in amanitin-treated cells double labeled with anti-m3G cap (I) and 3C5 (L) antibodies. Bar, 10 μ m.

essentially identical response was obtained after injection of α -amanitin at concentrations ranging from 10–100 μ g/ml. No such changes in snRNP labeling were seen in any uninjected cells or in the cells injected with solvent not containing α -amanitin (data not shown).

In summary, these data show that after cells are exposed

to α -amanitin the pattern of snRNP labeling changes such that spliceosomal snRNPs no longer concentrate in foci and no longer colocalize with p80 coilin. Instead, each of the U1, U2, U4/U6, and U5 snRNPs concentrate in large, brightly staining "speckles." These structures likely correspond to enlarged clusters of interchromatin granules. The widespread

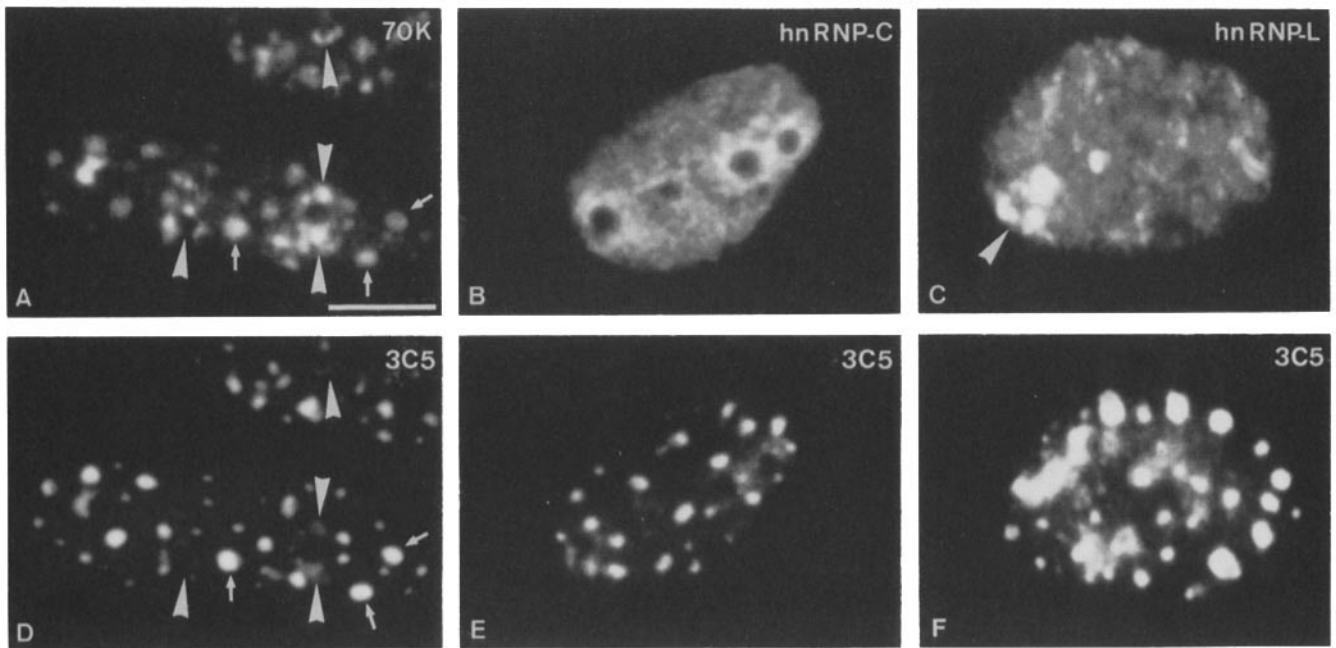


Figure 9. α -amanitin differentially affects snRNP and hnRNP staining. α -Amanitin-treated cells were Triton-extracted and double labeled with mAbs specific for U1snRNP 70K protein (A), hnRNP C (B), or hnRNP L (C) and monoclonal 3C5 (D, E, and F). The U1 snRNP is detected both in “speckles” (A, arrows) which colocalize with 3C5 (D, arrows) and in perinucleolar structures (A, arrowheads) which are recognized more weakly by the 3C5 antibody (D, arrowheads). Anti-hnRNP C and L antibodies show widespread nucleoplasmic staining. They do not concentrate in speckles (B and C) and do not colocalize with the 3C5 monoclonal (E and F). Note that the concentrated regions of hnRNP L staining persist after α -amanitin treatment (C, arrowhead) and show no apparent relation to the 3C5-stained “speckles” (F). Bar, 10 μ m.

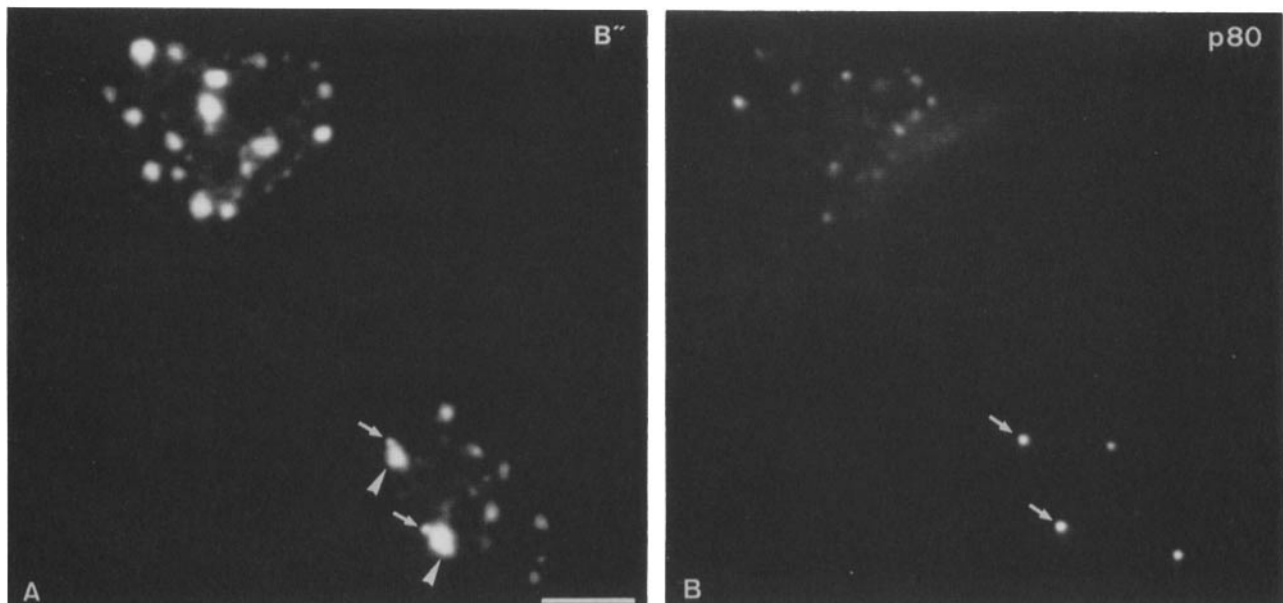


Figure 10. Microinjection of α -amanitin into HeLa cell nuclei. Cells were microinjected with α -amanitin (10 μ g/ml) and incubated for 1 h before Triton extraction and fixation. In injected cells the anti-B'' staining concentrates in large, bright “speckles” (A). Double labeling with anti-p80 coilin antibody (B) shows no colocalization in the large “speckles.” In some cells foci of p80 coilin staining are observed (lower cell) while in others the staining pattern appears fragmented (upper cell). Note that anti-B'' and anti-p80 coilin antibodies occasionally colocalize in foci adjacent to enlarged speckles (arrows). Bar, 10 μ m.

nucleoplasmic staining outside of "speckles" and coiled bodies which is normally seen in untreated cells is also greatly reduced or eliminated after α -amanitin treatment. This effect is particularly striking for U1 snRNP, which shows the highest level of widespread nucleoplasmic staining in untreated cells.

Discussion

In this study, the snRNP-rich nuclear foci which we had recently identified in mammalian nuclei are shown to be coiled bodies. In addition, we have established *in situ* labeling conditions which demonstrate that each of the U1, U2, U4/U6, and U5 snRNPs are also present throughout the nucleoplasm and are enriched in clusters of interchromatin granules. One conclusion from these studies therefore is that the punctate or "speckled" pattern seen by immunofluorescence in cells stained with anti-Sm or anti-m3G-Cap antibodies is not homogeneous, but rather includes at least two separate types of nuclear structures, *i.e.*, coiled bodies and interchromatin granules. At the EM level, coiled bodies and interchromatin granules are morphologically distinct (42). It is now apparent that they are also functionally distinct compartments, as snRNP association with coiled bodies and interchromatin granules is differentially affected by both actinomycin D and α -amanitin (Figs. 7–10). From our previous analyses the snRNPs in coiled bodies also appear more accessible to antisense probes, both *in situ* (15; and this study) and *in vivo* (16), than the same snRNP species present in interchromatin granules. These observations suggest that the snRNPs present in coiled bodies and interchromatin granules might be involved in different metabolic activities.

Previously, using antibodies specific for Sm proteins or m3G-Cap structures, it could not be distinguished whether all classes of snRNP, or only a sub-set, were present at any given nuclear location. This is an important point for considering possible sites in the nucleus where pre-mRNA splicing can occur, as each of these snRNPs is required *in vitro* for splicing and spliceosome assembly. The present study provides a clear demonstration that each species of spliceosomal snRNP is present in multiple nucleoplasmic locations. As a perfect colocalization is observed for the staining patterns of antisense probes and cognate mAbs specific for both U1 and U2 snRNPs, we can also now be confident that mature snRNP particles are being detected and not nascent snRNAs or pools of unassembled snRNP proteins. Unfortunately, in the case of mammalian U4/U6 or U5 snRNPs, no specific antibodies are currently available to use in conjunction with the antisense probes for double-labeling experiments. From our studies with the antisense probes alone however it appears that U4/U6 and U5 have a similar *in situ* distribution to U2 snRNP. In contrast, U1 snRNP shows an interesting quantitative difference in its distribution. Here, and in our previous *in situ* and *in vivo* studies (15, 16), we observe a larger fraction of U1 snRNP widely distributed throughout the nucleoplasm rather than concentrated in coiled bodies or "speckles." This distribution resembles that seen for the non-snRNP splicing factor U2AF and we have presented evidence that U1 and U2AF may exist in some form of common complex *in vivo* (16). Both actinomycin D and α -amanitin also affect U1 differently from U2, U4/U6, and U5. Interestingly, recent studies of snRNP distribution

in *Xenopus* oocytes also point to a difference in the organization of U1 relative to the other spliceosomal snRNPs (reviewed in reference 24). In oocytes, U1 is detected both in particles containing all species of snRNPs ("B snurposomes") and in distinct particles containing just U1 ("A snurposomes"). Data from *in vitro* splicing studies may point to a possible functional difference between U1 and the other snRNPs; *e.g.*, U1 alone binds to 5' splice sites, is able to bind pre-mRNA in the absence of ATP, kinetically is the first snRNP to assemble on splicing substrates and also appears to bind less stably to the spliceosome than the other snRNP species (reviewed in references 38, 53). It seems plausible therefore that the difference we have observed in mammalian cells between U1 snRNP distribution and that of U2, U4/U6, and U5 reflects a genuine difference in its functional role *in vivo*.

The data presented here show that snRNPs are no longer found concentrated in coiled bodies when cells are treated with the transcription inhibitors α -amanitin or actinomycin D, or when cells are heat shocked (Figs. 7–10). The association of snRNPs with coiled bodies may therefore depend directly on the presence of newly synthesized pre-mRNA. These observations argue in favor of coiled bodies playing an active role in the metabolism of nuclear pre-mRNA. This is consistent with our previous proposal that the snRNP-rich nuclear foci (coiled bodies), represent nuclear organelles or compartments in which a specific part of the pre-mRNA maturation pathway takes place (16). An important corollary of these data is that changes in metabolic activity within a cell can cause changes in the relative concentrations of snRNP present in different nuclear structures. It is therefore possible that different patterns of snRNP distribution may be found in other cell types which differ in their metabolic activity from the HeLa cells used in this study. In this regard it will be interesting to compare how snRNP distributions are affected when cells in culture are induced to differentiate and undergo major changes in their metabolism and pattern of gene expression.

The coiled body is a nuclear structure that was originally seen in 1903 by Ramon y Cajal, who called it the "accessory body" (49). Subsequent ultrastructural studies identified nuclear "accessory bodies" (29) and "coiled bodies" (42) in the electron microscope and these structures were later confirmed as being the accessory bodies described by Ramon y Cajal (35, 56). Coiled bodies are typically described at the ultrastructural level as oval to spherical inclusions, ranging in size from 0.3–0.9 μm and giving the appearance of bundles of "knotted threads." They also appear to be highly conserved in evolutionary terms, showing a similar appearance in eukaryotes as diverged as plants and mammals (43, 57). While an extensive literature exists describing the appearance and occurrence of coiled bodies, few functional studies have been carried out to address their biological role. As coiled bodies are often observed close to nucleoli, particularly in neuronal cells, it has frequently been suggested that they could play a role in nucleolar function (29, 42, 50, 56, and 57). In contrast, Schultz (57) speculated that coiled bodies may correspond to assemblies of spliceosomes. Fakan *et al.* (21) observed at the EM level that coiled bodies were among the nuclear structures which were preferentially labeled by anti-Sm and anti-hnRNP antibodies. This is consistent with them being involved in pre-mRNA metabolism.

Furthermore, studies on plant coiled bodies have shown that they are sites of acid phosphatase activity, which is also suggestive of an RNA processing activity (43).

Although the data in this study strongly support the view that coiled bodies are involved in pre-mRNA metabolism, they do not prove that coiled bodies are actual sites of pre-mRNA splicing. The observed concentration of snRNPs and the splicing factor U2AF in coiled bodies, and the apparent dependence of this association on the production of nascent transcripts, is also consistent with them having a role in pre-mRNA metabolism before or after splicing has taken place. For example, coiled bodies could represent organelles where snRNPs are pre-assembled, together with other splicing factors. Alternatively, they could be sites where introns are debranched and/or degraded after splicing, where snRNPs are recycled from post-splicing complexes or where intron and mRNA splicing products are sorted out as part of a nuclear export pathway. We note that if coiled bodies play a role in some post-splicing event, then any splicing factors which do not remain bound to spliceosomes after exon ligation is completed would not accumulate there. This is a possible explanation why the splicing factor SC-35 is not detected in coiled bodies (16). If coiled bodies do represent actual sites of pre-mRNA splicing, it is also conceivable that they are specialized structures dedicated to the processing of a specific sub-set of pre-mRNAs. In a recent study Wang et al. (71) showed that when in vitro synthesized pre-mRNAs were injected into mammalian cells they became localized in punctate nuclear structures, including those stained by anti-Sm and anti-SC-35 antibodies. It will be interesting now to determine whether any of these structures in which exogenous pre-mRNAs concentrate correspond to coiled bodies.

Experiments are currently in progress to purify coiled bodies from mammalian cells and characterize biochemically their composition. Hopefully such studies will lead to a clearer understanding of the in vivo role of coiled bodies and their significance for nuclear pre-mRNA metabolism.

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