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Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery

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The organization of the major snRNP particles in mammalian cell nuclei has been analysed by *in situ* labelling using snRNA-specific antisense probes made of 2'-OMe RNA. U3 snRNA is exclusively detected in the nucleolus while all the spliceosomal snRNAs are found in the nucleoplasm outside of nucleoli. Surprisingly, U2, U4, U5 and U6 snRNAs are predominantly observed in discrete nucleoplasmic foci. U1 snRNA is also present in foci but in addition is detected widely distributed throughout the nucleoplasm. An anti-peptide antibody specific for the non-snRNP splicing factor U2AF reveals it to have a similar distribution to U1 snRNA. Co-localization studies using confocal fluorescence microscopy prove that U2AF is present in the snRNA-containing foci. Antibody staining also shows the foci to contain snRNP-specific proteins and m3G-cap structures. The presence of major components of the nuclear splicing apparatus in foci suggests that these structures may play a role in pre-mRNA processing.

Key words: mammalian nucleus/pre-mRNA splicing/snRNA/2'-OMe RNA/*in situ* labelling

Introduction

Small nuclear RNAs, commonly designated U snRNAs due to their high uridylic acid content, are present in all eukaryotic cells. U snRNAs account for ~1% of total RNA in mammalian cells and are functionally involved in the maturation of pre-mRNAs, pre-rRNAs and possibly other RNA processing events (for recent reviews see Reddy and Busch, 1988; Steitz *et al.*, 1988; Zieve and Sauterer, 1990).

Molecular genetic and *in vitro* biochemical approaches have shown that U1, U2, U4/U6 and U5 snRNAs are integral components of 'spliceosomes', i.e. the molecular apparatus responsible for pre-mRNA splicing (reviewed by Maniatis and Reed, 1987; Sharp, 1987; Guthrie and Patterson, 1988; Steitz *et al.*, 1988; Lamond *et al.*, 1990). In contrast, U3, another abundant snRNA, is restricted to the nucleolus and has been recently demonstrated to function in the first step of pre-ribosomal processing (Kass *et al.*, 1990).

In cells, snRNAs are present as ribonucleoprotein particles, the so-called snRNPs. Each snRNP consists of one or several snRNAs and a defined set of associated polypeptides. The snRNAs U1, U2 and U5 exist as monomeric RNP particles, while U4 and U6 are found together in one complex (Lerner and Steitz, 1979; Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Blencowe *et al.*, 1989). Recently U4/U6 and U5 snRNAs have also been shown to form an ATP-dependent U4/U5/U6 triple snRNP complex (Konarska and Sharp, 1987; Cheng and Abelson, 1987; Black and Pinto, 1989). A major advance in understanding snRNP structure and function followed the discovery that some antibodies found in the sera of patients with autoimmune connective tissue diseases are directed against snRNP proteins (Lerner and Steitz, 1979; Lerner *et al.*, 1981). Two major distinct types of autoimmune sera were identified: sera of the 'Sm' type immunoprecipitate all snRNP particles containing U1, U2, U4/U6 and U5 RNAs, while anti-(U1)RNP sera react only with U1-containing particles (Fischer *et al.*, 1983; Pettersson *et al.*, 1984). Further immunological studies have contributed to the identification of at least 14 snRNP polypeptides. Some of these proteins are common to all snRNPs, while others are unique to specific particles (reviewed by Lürhmann, 1988). Both classes of polypeptides share common epitopes, as demonstrated by cross-reactivity with monoclonal antibodies (Lerner *et al.*, 1981; Pettersson *et al.*, 1984; Reuter and Lührmann, 1986; Reuter *et al.*, 1986; Williams *et al.*, 1986). Antibodies have also been raised against modified bases found in snRNPs, making possible the recognition of RNA moieties in RNP particles (Lürhmann *et al.*, 1982; Smith and Eliceiri, 1983). However, as these modified bases are present in several snRNAs and tRNAs, such antibodies cannot be used to identify unique RNA species.

A highly specific tool for visualizing the distribution of individual snRNAs within intact nuclei could provide important insights into the mechanism and spatial organization of the splicing reaction *in vivo*. We have recently described the use of nuclease resistant, 2'-OMe RNA oligonucleotides as antisense probes for studying the structure and function of specific human snRNPs (Barabino *et al.*, 1989, 1990; Blencowe *et al.*, 1989; Lamond *et al.*, 1989). Here we report the development of an *in situ* hybridization procedure using biotinylated 2'-OMe RNA oligonucleotides to label the major snRNAs in mammalian cells. The presence of biotin allows visualization of the oligonucleotide binding sites through secondary labelling with an avidin-coupled fluorochrome. We show the pattern of snRNA localization obtained using the 2'-OMe RNA antisense probes and compare this with immunofluorescence staining using antibodies which recognize the snRNA-specific m3G-cap structure, snRNP proteins and the non-snRNP splicing factor, U2AF.

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Results

Specificity of 2′-OMe RNA probes

In order to study the *in situ* localization of the major snRNAs in mammalian cells, a battery of specific antisense probes was used. We have previously shown that biotinylated oligonucleotides made of 2′-OMe RNA bind specifically to targeted snRNAs *in vitro* and can therefore be used to affinity select snRNP particles from HeLa cell nuclear extracts using streptavidin chromatography (Barabino *et al.*, 1989, 1990; Blencowe *et al.*, 1989). In these studies antisense probes were reported that bind specifically to U1, U2, U4 and U6 snRNAs. Affinity selection assays demonstrating the specificity of binding of these antisense probes to snRNAs in HeLa cell nuclear extracts are shown in Figure 1A. Also shown here is a new probe that binds specifically to the other major spliceosomal snRNA, U5 (Figure 1A, lane 5). A biotinylated 2′-OMe RNA oligonucleotide complementary to the nucleolar snRNA U3 was also synthesized and tested for specificity (Figure 1B). This shows that only U3 snRNA is selected by the anti-U3 probe and not the spliceosomal snRNAs (i.e. U1, U2, U4, U5 and U6), or another nucleolar snRNA, U8 (Figure 1B, lanes 2 and 3). Control experiments show that no U3 snRNA is selected in the absence of an antisense oligonucleotide (Figure 1B, lanes 4 and 5), or by an anti-U2 snRNA probe (Figure 1B, lanes 6 and 7).

In situ localization of the major U-snRNAs

Having established that the 2′-OMe RNA oligonucleotide probes hybridize specifically to U1, U2, U3, U4, U5 and U6 snRNAs *in vitro*, they were then used to analyse the *in situ* distribution of each snRNA in mammalian cell nuclei (Figure 2). Of the different fixation/permeabilization strategies tested, optimal hybridization efficiency was obtained after Triton X-100 extraction and mild formaldehyde fixation. Similar results were obtained with Triton extraction times varying from 30 s to 3 min, or replacing CSK buffer by buffer A (see Materials and methods).

As expected, the anti-U3 snRNA probe exclusively labelled nucleoli (Figure 2A), while all the spliceosomal snRNA labelling was nucleoplasmic and excluded nucleoli (Figure 2, B,C,G,H and I). Two distinct labelling patterns were observed with the spliceosomal snRNA probes. In the case of U1 snRNA, labelling was distributed throughout the nucleoplasm, excluding nucleoli, but often forming a perinucleolar rim (Figure 2B). In contrast, the U2 snRNA probe revealed a striking pattern of bright 'spots', or foci (Figure 2C). This was observed using both formaldehyde–methanol fixed and Triton pre-extracted cells, although the latter method gave more efficient labelling. Antisense oligonucleotides targeted to each of U4, U5 and U6 snRNAs also labelled a similar pattern of bright spots (Figure 2, G–I). In each case the labelling was concentrated in these foci. As shown in Table I, the mean number of foci per nucleus labelled with each of the U2, U4, U5 and U6 snRNA probes is apparently identical. Simultaneous labelling with three of these antisense probes (anti-U2, U5 and U6) did not increase the mean number of spots per nucleus detected (Table I). This makes it very likely that the same structures are being labelled by each of the U2, U4, U5 and U6 snRNA-specific probes.

The different labelling patterns discussed above were also confirmed by confocal fluorescence microscopy. This technique provides an improvement in contrast through the reduction of out-of-focus 'blur'. Confocal microscopy demonstrates that the anti-U1 snRNA oligonucleotide probe also labels discrete spots akin to the foci detected with the other probes (Figure 2J). This labelling of foci by the anti-U1 snRNA probe tends to be veiled by the intense nucleoplasmic staining seen using conventional fluorescence microscopy (cf. Figure 2, B and J). Taken together, these results point to the presence of discrete foci within the nucleoplasm where

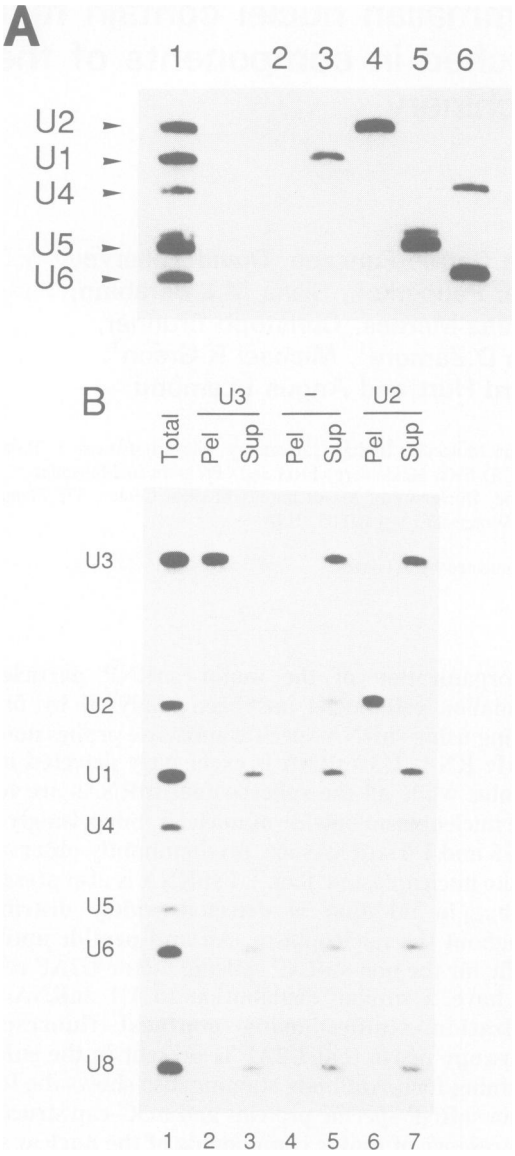

Fig. 1. Affinity selection of targeted snRNAs. Biotinylated 2′-OMe RNA oligonucleotides were incubated in HeLa nuclear splicing extracts, affinity selected using streptavidin agarose beads and the selected RNA species analysed by Northern hybridization. (**A**) Selection of spliceosomal snRNAs using antisense probes to U1 (lane 3), U2 (lane 4), U5 (lane 5) and U6 (lane 6). A negative control with no oligonucleotides is shown in lane 2. Lane 1 is a marker lane with total HeLa nuclear RNA. (**B**) Selection of the nucleolar snRNA U3. Lane 1 shows total HeLa nuclear RNA. Lanes 2 and 3 show RNA recovered from pellet and supernatant fractions respectively after affinity selection using an antisense probe complementary to U3 snRNA. Lanes 4 and 5 show pellet and supernatant fractions for a no oligonucleotide negative control selection and lanes 6 and 7 show the pellet and supernatant fractions from a positive control selection using the anti-U2 oligonucleotide probe.

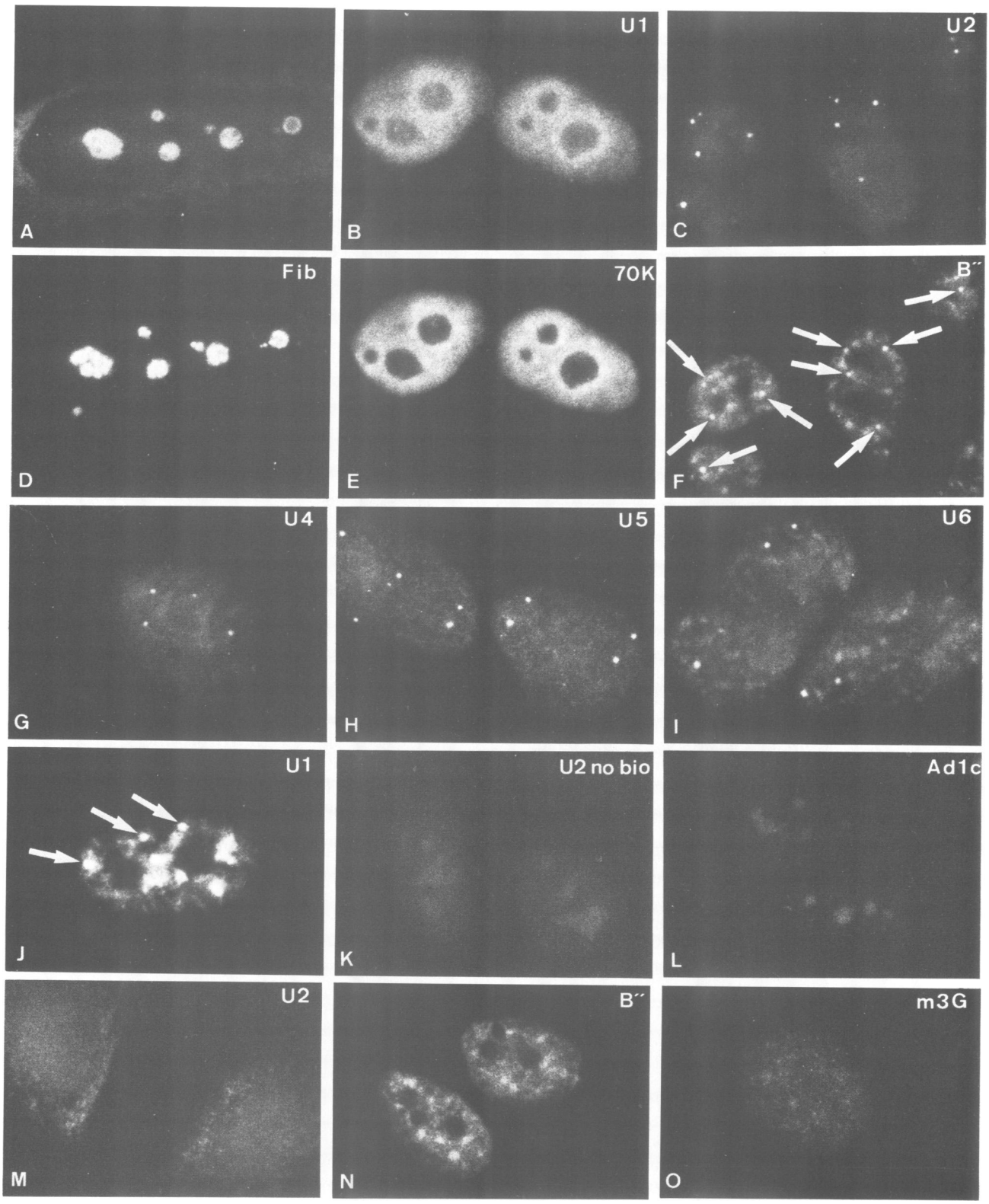

Fig. 2. *In situ* hybridization using antisense 2′-OMe RNA oligonucleotide probes in whole cells. All cells were permeabilized with 0.5% Triton X-100 before formaldehyde fixation, except those depicted in panels (A) and (D), which were formaldehyde fixed and methanol extracted. Double-labelling experiments demonstrate co-localization of anti-U3, U1 and U2 snRNA probes (**A**, **B**, **C**) with, respectively, fibrillarin, anti-70K and B″ antibodies (**D**, **E**, **F**). In addition to the U2 snRNA-labelled spots (arrows), the anti-B″ antibody stains numerous nucleoplasmic speckles (F). Oligonucleotide probes directed against U4, U5 and U6 snRNAs (**G**, **H**, **I**) show a labelling pattern similar to that obtained with the anti-U2 snRNA probe, i.e. discrete nucleoplasmic spots. Confocal fluorescence microscopy allows identification of 'spots' labelled with the anti-U1 snRNA oligonucleotide (**J**, arrows). Pre-incubation with non-biotinylated anti-U2 snRNA (150 pM/μl) results in a loss of signal for biotinylated anti-U2 snRNA (**K**). An RNA probe complementary to the first intron of the adenovirus major late transcript shows only weak background nucleolar staining (**L**). Digestion with RNase A resulted in a complete loss of nuclear spots detectable with the anti-U2 snRNA oligonucleotide probe (**M**), anti-B″ antibody (**N**) and anti-m3G-cap antibody (**O**). Despite the absence of spots, the anti-B″ antibody reveals a speckled nucleoplasmic staining (**N**). Magnification: (A–O) 1200×; (I) 2800×.

Table I. Quantification of foci number per nucleus

Probe	No. of foci	Significance level
anti-U2 snRNA	3.72 (1.48)	0.284
anti-U4 snRNA	3.24 (1.49)	0.151
anti-U5 snRNA	3.85 (1.77)	0.156
anti-U6 snRNA	3.60 (1.57)	0.720
anti-U2+U5+U6 snRNAs	3.69 (1.52)	0.418
anti-B″ protein[a]	3.53 (1.28)	

The mean number of foci per nucleus is shown for each probe with the standard deviation indicated in parentheses. Each mean is calculated from a sample size of 100 nuclei. A paired Student's *t*-test was applied to compare the mean number of foci labelled by each 2′-OMe RNA oligonucleotide with the mean number of foci labelled by the anti-B″ antibody. The null hypothesis ($\mu 1 = \mu 2$) is not rejected at a significance level of 0.05.

[a]The identification of foci stained by the anti-B″ antibody was initially done on the basis of their distinct morphology and subsequently confirmed by co-localization with an anti-U2 snRNA oligonucleotide probe.

most of the U2, U4/U6 and U5 snRNAs are detected. U1 snRNA is also present in foci but in addition occurs widely distributed throughout the nucleoplasm.

As a control for the *in situ* binding specificity of the antisense probes, competition experiments with non-biotinylated anti-snRNA oligonucleotides were performed. Pre-binding with a non-biotinylated version of the anti-U2 snRNA probe, followed by subsequent binding of the biotinylated oligonucleotide, resulted in a specific loss of signal from the nucleoplasmic spots, leaving only weak, background staining (Figure 2K). However, after pre-blocking with the non-biotinylated anti-U2 oligonucleotide, foci could still be detected using probes targeted to either U4, U5 or U6 snRNAs (data not shown). A similar specific competition of labelling was observed when pre-blocking was done with a non-biotinylated version of the anti-U6 snRNA probe (data not shown). No specific staining with avidin–fluorescein was observed after incubation of cells with non-biotinylated 2′-OMe RNA oligonucleotides (data not shown). Control experiments using a non-snRNA targeted 2′-OMe RNA probe (complementary to the first intron of the adenovirus major late transcript), which does not bind *in vitro* to any of the major snRNAs (Ryder *et al.*, 1990), showed only weak, background nucleolar staining (Figure 2L). In order to confirm that the labelling obtained with the antisense oligonucleotides results from the probes binding to RNA, control experiments were done using RNase treated cells. After digestion of cells with RNase A, no nuclear foci were detected with the anti-U2 snRNA probe, although a faint background of widespread nuclear staining remained (Figure 2M). Foci were, however, detected in control cells incubated in the absence of nuclease (data not shown).

These various control experiments underline the specificity of labelling by the antisense 2′-OMe RNA oligonucleotides. The weak background nuclear staining probably reflects a low level of nonspecific binding. The background staining, especially at high probe concentrations, tends to be most pronounced in the nucleolus. This is most probably due to the extremely high RNA content in this organelle. It should also be noted that increasing either the concentration of oligonucleotide, or the time of incubation with fluorescein-conjugated avidin, led to higher background nuclear staining but never produced labelling of additional foci, or 'spot-like' structures. We conclude that the 2′-OMe RNA probes are binding specifically to targeted snRNA sequences *in situ* and thus are revealing sites of nuclear snRNA localization.

Correlation of antisense labelling of snRNA with antibody staining of snRNP proteins

To assess whether the observed snRNA localizations correspond to snRNPs, double-labelling experiments using anti-snRNP protein antibodies were performed. A purified anti-peptide antibody raised against human fibrillarin (Hurt,E.C., Jansen,R.P., Kern,H., Lehtonen,H., Carmo-Fonseca,M., Lapeyre,B. and Tollervey,D., submitted) was used to visualize a U3 snRNA associated protein. Fibrillarin, which was originally identified by autoimmune sera as a nucleolar protein specific for the fibrillar compartment, has recently been characterized as a U3 snRNA protein (Parker and Steitz, 1987). As shown in Figure 2 (A and D), both the anti-U3 snRNA oligonucleotide probe and the anti-fibrillarin antibody exclusively label nucleoli. As discussed above, the more diffuse staining of the nucleolus with the RNA probe compared with the antibody may be caused by a background of nonspecific probe binding due to the very high concentration of pre-rRNA in the nucleolus.

Labelling by the anti-U1 snRNA oligonucleotide co-localized with the immunostaining pattern of both a monoclonal anti-70K antibody (Figure 2, cf. B and E) and autoimmune anti-(U1)RNP serum (data not shown). In each case a diffuse nucleoplasmic staining was observed, which differs from the 'speckled' staining pattern previously reported for these antibodies (Spector, 1984; Verheijen *et al.*, 1986). This difference is due to the fixation procedure as will be discussed below (cf. Figure 3G). For U2 snRNP double-labelling, experiments were performed using the anti-U2 snRNA oligonucleotide and both a monoclonal antibody directed against the U2 snRNP-specific B″ polypeptide (Figure 2, cf. C and F) and anti-Sm polyclonal antibodies (data not shown). Discrete, bright 'spots' were labelled by both antibodies (arrows in Figure 2F). The same mean number of spots per nucleus was detected by the anti-B″ antibody as by the antisense probes specific for U2, U4, U5 and U6 snRNAs (Table I). However, in contrast with the antisense oligonucleotide labelling, antibody staining was not restricted to the spots. In addition, a less intense widespread labelling of the nucleoplasm in a 'speckled' pattern was also observed. This speckled staining pattern has been reported previously, but without identification of the foci as distinct structures (Spector, 1984; Nymann *et al.*, 1986). As shown below using confocal microscopy (Figure 4), these antibody-labelled, bright spots co-localize with the foci detected by the anti-U2 snRNA probe. This indicates that the foci correspond to sites where U2 snRNPs concentrate.

In RNase treated cells, there is no labelling of foci by the anti-B″ antibody, although we note that the speckled staining pattern persists (Figure 2N). No specific labelling in these RNase treated cells is detected using either antisense probes (cf. Figure 2M) or the snRNA-specific anti-m3G-cap antibody (Figure 2O). This indicates that the speckled component of the staining pattern observed with the B″ antibody is distinct from the brightly-labelled foci.

Co-localization of snRNAs with snRNP-specific proteins, m3G-cap structures and the non-snRNP splicing factor U2AF

The fact that the antisense probes for U2, U4, U5 and U6 snRNAs detect the same mean number of foci per nucleus

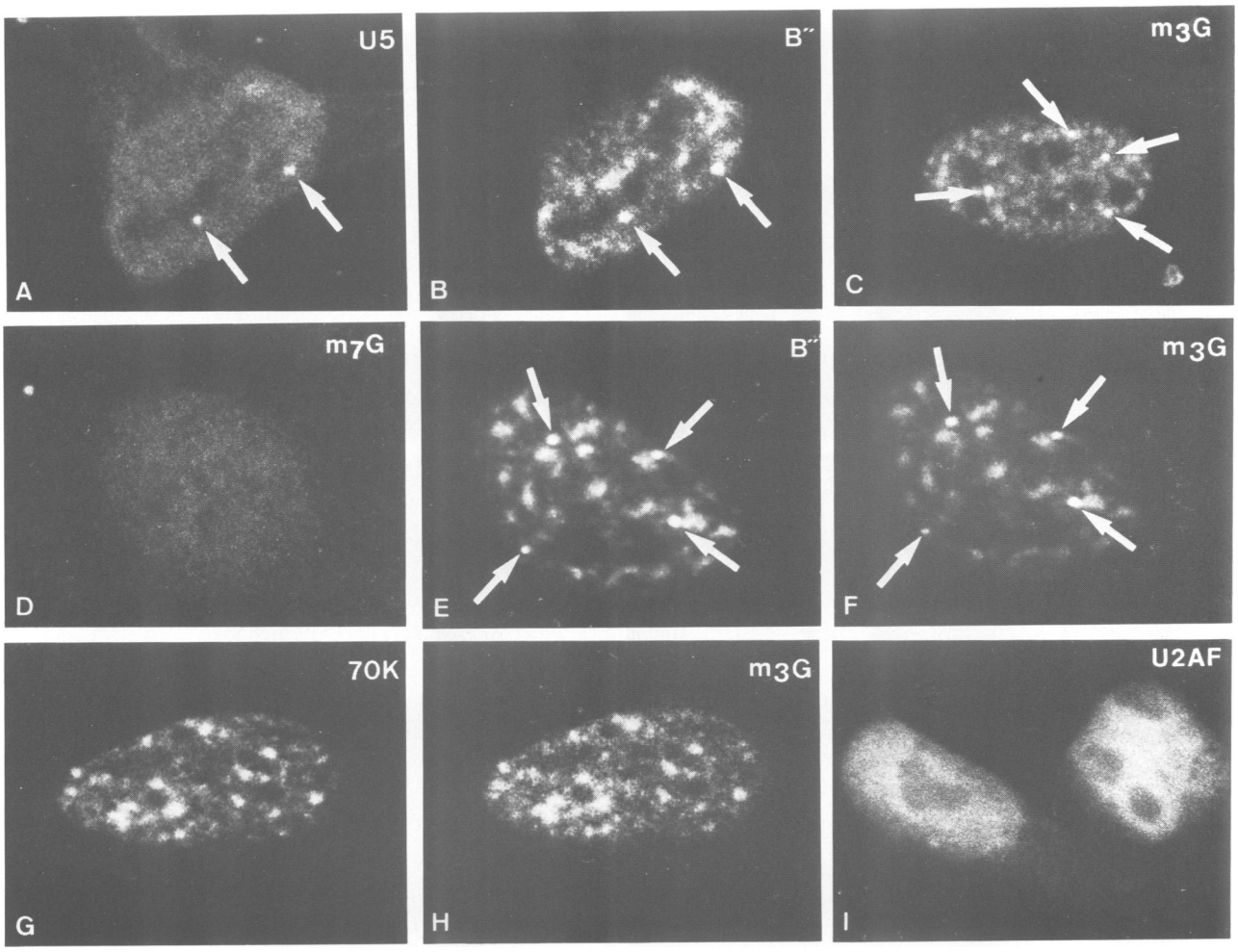

Fig. 3. Co-localization of the anti-U5 snRNA labelled spots (**A**) with the anti-B″ antibody staining (**B**). Labelling of HeLa cells with anti-m3G-cap antibody reveals a speckled nucleoplasmic staining and discrete bright spots (**C**, arrows). This staining pattern is completely abolished in the presence of 25 mM m7G (**D**). This cap analogue is known to block the antibodies when used as a free base, even though the antibodies show no reactivity against m7G-cap structures (Lührmann *et al.*, 1982; Reuter *et al.*, 1984). Both foci (arrows) and nucleoplasmic speckles labelled by the anti-m3G-cap antibody co-localize with the anti-B″ antibody staining (**E** and **F**). Upon methanol–acetone fixation, anti-U1 snRNP-specific antibodies reveal a speckled nucleoplasmic staining pattern which co-localizes with anti-m3G-cap labelling (**G** and **H**). Anti-U2AF antibody stains the nucleoplasm with a diffuse pattern, excluding nucleoli (**I**). Magnification: (A, B, E–H) 2100× (confocal fluorescence microscopy); (C, D and I) 1200× (conventional fluorescence microscopy).

implies that all these snRNPs are co-localized in the same spot-like structures. As the B″-specific antibody also labels the same mean number of foci and co-localizes with the anti-U2 snRNA probe, this predicts that the foci detected by the anti-B″ antibody should also co-localize with each of the anti-U4, U5 and U6 snRNA probes. This is indeed observed. Co-localization of the anti-U5 oligonucleotide probe with the anti-B″ antibody is shown in Figure 3A and B. Identical results were obtained with the anti-U4 and anti-U6 snRNA probes (data not shown). This provides strong evidence that each of these snRNPs are present in the foci.

If the major snRNPs concentrate in the foci then those structures should also be brightly labelled by antibodies directed against the snRNA-specific 2,2,7-trimethyl-guanosine (m3G)-cap structure. In agreement with previous studies (Reuter *et al.*, 1984), we observe that both polyclonal and monoclonal anti-m3G-cap antibodies show a widespread, speckled staining of the nucleoplasm (Figure 3C), which is completely abolished in the presence of m7G as a competitor (Figure 3D). Within this staining pattern however brighter spots are apparent (arrows in Figure 3C). These brighter spots also co-localize with the foci labelled by the anti-snRNA oligonucleotide probes (Figure 4D and other data not shown). Both components of the anti-B″ antibody staining pattern, i.e. the bright spots and the widespread speckled labelling, closely co-localize with the anti-m3G-cap labelling (Figure 3, compare E and F). However, a significant difference is observed when the same antibodies are used to stain RNase treated cells (Figure 2, N and O). The anti-m3G-cap antibody shows no specific nuclear staining after RNase treatment while the anti-B″ antibody still gives the widespread speckled staining but no longer labels bright spots.

It has previously been reported that the anti-m3G-cap staining pattern also co-localized with that obtained using both anti-Sm and anti-(U1)RNP antibodies (Reuter *et al.*, 1984). As we observed a more diffuse, rather than speckled, staining pattern with both the anti-U1 snRNA oligonucleotide probe and U1 snRNP-specific antibodies (Figure 2B and E),

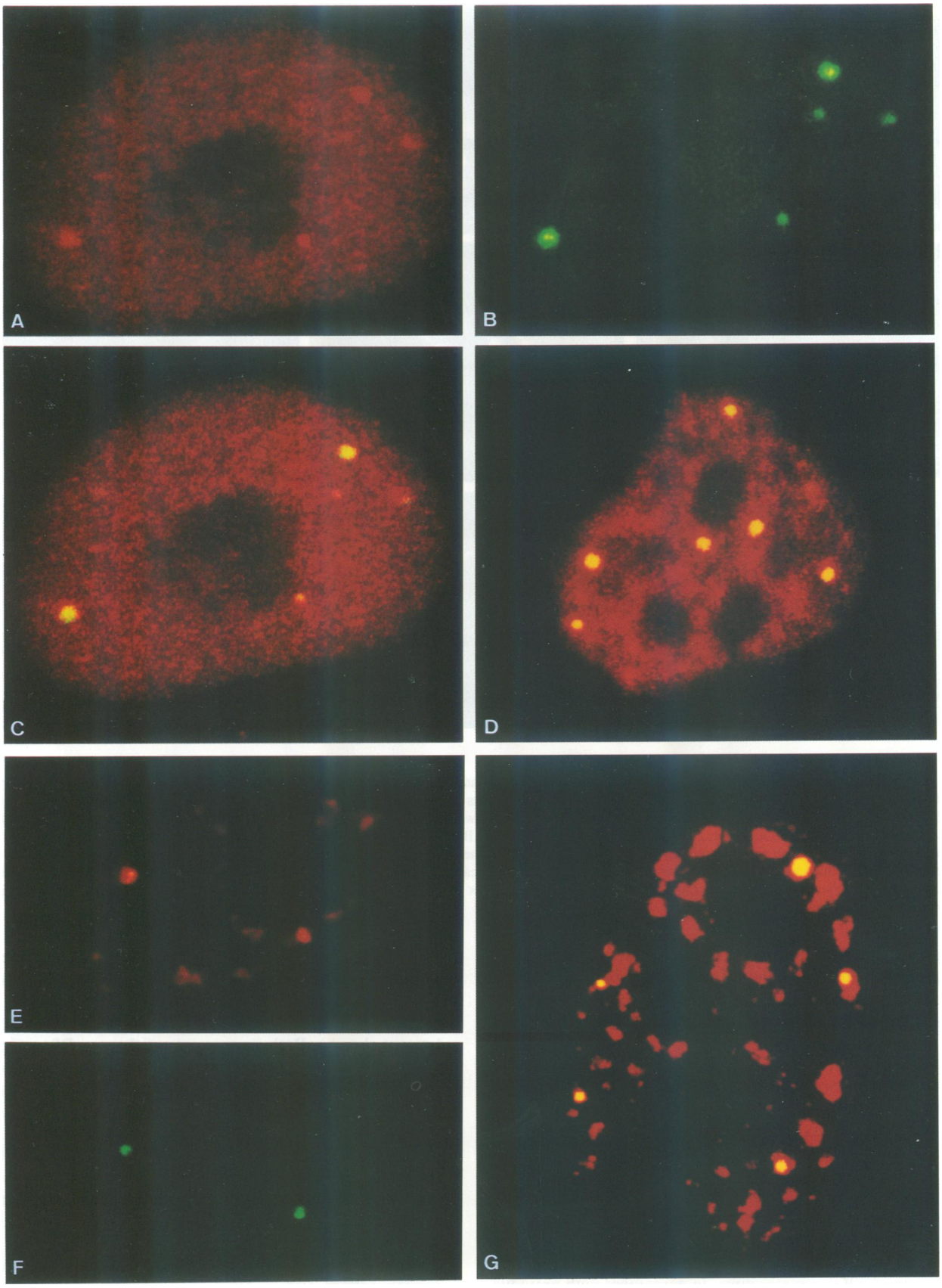

Fig. 4. Pseudo-coloured confocal images of HeLa cells double-labelled with anti-U2 snRNA 2′-OMe RNA oligonucleotide probes (green) and antibodies (red) directed against U2AF (**A**, **C**), m3G-cap (**D**) and B″ (**E**, **G**). Projections of each confocal series were calculated and overlapped (C, D, G). Co-localization sites are seen as yellow structures (green/red overlay). In (A), (B) and (C) the same cell is shown. This cell was microinjected with anti-U2AF antibody prior to Triton extraction and paraformaldehyde fixation. Anti-U2AF binding sites were visualized with a

we repeated these antibody labelling experiments using a methanol–acetone fixation protocol instead of Triton pre-extraction. As shown in Figure 3G, this alternative fixation protocol causes a major change in the U1 snRNP labelling pattern. The diffuse, widespread nucleoplasmic staining obtained after Triton pre-extraction is replaced by a speckled pattern which now co-localizes with that obtained using the anti-m3G-cap antibody (Figure 3, compare G and H). Note that labelling of Triton pre-extracted cells with these same antibodies does not show co-localization (cf. Figures 2E and 3C), although the anti-U1 snRNA oligonucleotide and U1-specific antibody staining do co-localize (Figure 2, cf. B and E).

U2AF is a non-snRNP protein factor which has recently been purified and shown to bind to pre-mRNAs at an early stage of spliceosome assembly (Ruskin *et al.*, 1988; Zamore and Green, 1989). The accompanying paper by Zamore and Green describes the characterization of anti-peptide antibodies specific for U2AF and demonstrates that immunodepletion of U2AF inhibits splicing *in vitro*. Splicing activity is restored to depleted extracts by addition of affinity purified U2AF, demonstrating that this is an essential splicing factor. We have therefore used these antibodies specific for U2AF to determine whether this factor co-localizes in the snRNP-containing foci. Using conventional immunofluorescence microscopy the anti-U2AF antibody shows intense nucleoplasmic staining, excluding nucleoli, in a pattern similar to that observed with the anti-U1 snRNA oligonucleotide probe (cf. Figures 3I and 2B). Although focus-like structures were observed in some experiments, the results were variable, depending on the method of fixation (data not shown). To minimize detection problems associated with fixation procedures, we therefore microinjected the anti-U2AF antibody directly into unfixed HeLa cells. As shown below, this makes it clear that the anti-U2AF antibody also labels the snRNP-containing foci (Figure 4 A–C).

In order to demonstrate conclusively that the foci detected by the antisense 2′-OMe RNA probes correspond to the same structures labelled with anti-m3G-cap, anti-U2AF and anti-B″ antibodies, images of double-labelled cells were recorded using the confocal fluorescence microscope and subsequently overlaid (Figure 4). Pseudo-green and red colour was given to oligonucleotide and antibody staining respectively. Co-localization sites appear yellow on such overlays. This shows that the foci labelled by the anti-U2 snRNA probe are also immunostained by antibodies directed against the splicing factor U2AF (Figure 4A–C), m3G-cap structures (Figure 4D) and B″ protein (Figure 4E–G).

Particular emphasis was directed towards characterizing features of the snRNP-containing foci. These were found to be regular, round-shaped structures on both horizontal and vertical optical sections (cf. Figure 4E and F, and data not shown). They are also detected in different optical planes and, in general, do not reach the nuclear periphery.

The snRNP-containing foci associate with the nuclear matrix

To characterize in more detail the snRNP-containing foci, we tested whether they were retained in nuclear matrix preparations (Figure 5A and B). To prepare nuclear matrices, cells were extracted *in situ* with nonionic detergent, digested with RNase-free DNase I and then further extracted with 0.25 M ammonium sulphate. Removal of chromatin from the nucleus was confirmed by DNA staining. Subsequent labelling with the anti-U2 oligonucleotide probe showed that the foci could still be detected (Figure 5A). They were also labelled by the anti-m3G antibody (data not shown). The foci labelled in nuclear matrix preparations appear visible as dense structures when viewed by phase contrast (Figure 5B). We observed that after extraction of nuclei with higher salt buffers, such as are used for the preparation of *in vitro* splicing extracts (Dignam *et al.*, 1983; Barabino *et al.*, 1990), the labelling pattern obtained with antisense probes is more widespread and not restricted to foci (Figure 5C). Based on these results we infer that the snRNP-containing foci are sensitive to high salt and associated in some way with the nuclear matrix.

Actinomycin D treatment changes the pattern of snRNP labelling

Actinomycin D, a potent inhibitor of RNA transcription, has long been known to affect nuclear and nucleolar structure (Simard *et al.*, 1974). Electron microscopic studies from several groups have shown that this drug causes a condensation of chromatin into aggregates, clumping of interchromatin granules and segregation of nucleolar components. One would thus expect that the distribution of snRNPs might also be affected by actinomycin treatment.

The organization of snRNPs in actinomycin treated cells was therefore analysed using both anti-snRNA oligonucleotide probes and snRNP protein-specific antibodies. After a 2 h exposure to actinomycin, labelling with anti-U2, U4, U5 and U6 snRNA oligonucleotide probes revealed the persistence of a spot-like staining pattern (Figure 5D and other data not shown). However, the size distribution of individual foci appears more variable. Double-labelling experiments showed that the B″ antigen still co-localized with the foci after actinomycin treatment (Figure 5G). A novel and striking effect of actinomycin was observed on the distribution of U1 snRNP. Labelling with both the anti-U1 snRNA oligonucleotide probe and the anti-U1 RNP antibody showed that U1 snRNP became capped around the remnants of the nucleolus (Figure 5E and H). In contrast with the other spliceosomal snRNPs, therefore, we have no evidence that U1 snRNP is present in foci following actinomycin treatment.

Upon blocking the transcription of rRNA genes by either drugs or microinjection of antibodies to RNA polymerase I, the normal nucleolar structure disintegrates and numerous small spherical aggregates can be seen at the nucleolar periphery and dispersed through the nucleoplasm (reviewed by Scheer and Benavente, 1990). These extranucleolar bodies are composed of tightly packed fibrillar material and, as shown by antibody staining, contain fibrillarin. Such phenomena are demonstrated in Figure 5I: anti-fibrillarin staining reveals numerous spherical structures in the nucleoplasm, and some at the edge of remnant nucleoli (as identified by phase contrast). Labelling with the anti-U3 snRNA oligonucleotide probe produced a similar pattern

secondary antibody coupled to Texas Red. Double labelling was subsequently performed with a biotinylated anti-U2 snRNA oligonucleotide and fluorescein–avidin. In (E) and (F) single frames from a confocal series are depicted. Due to the fact that spots occur at different optical planes, their actual number can only be visualized in projections of the whole confocal series. Magnification: 4000×.

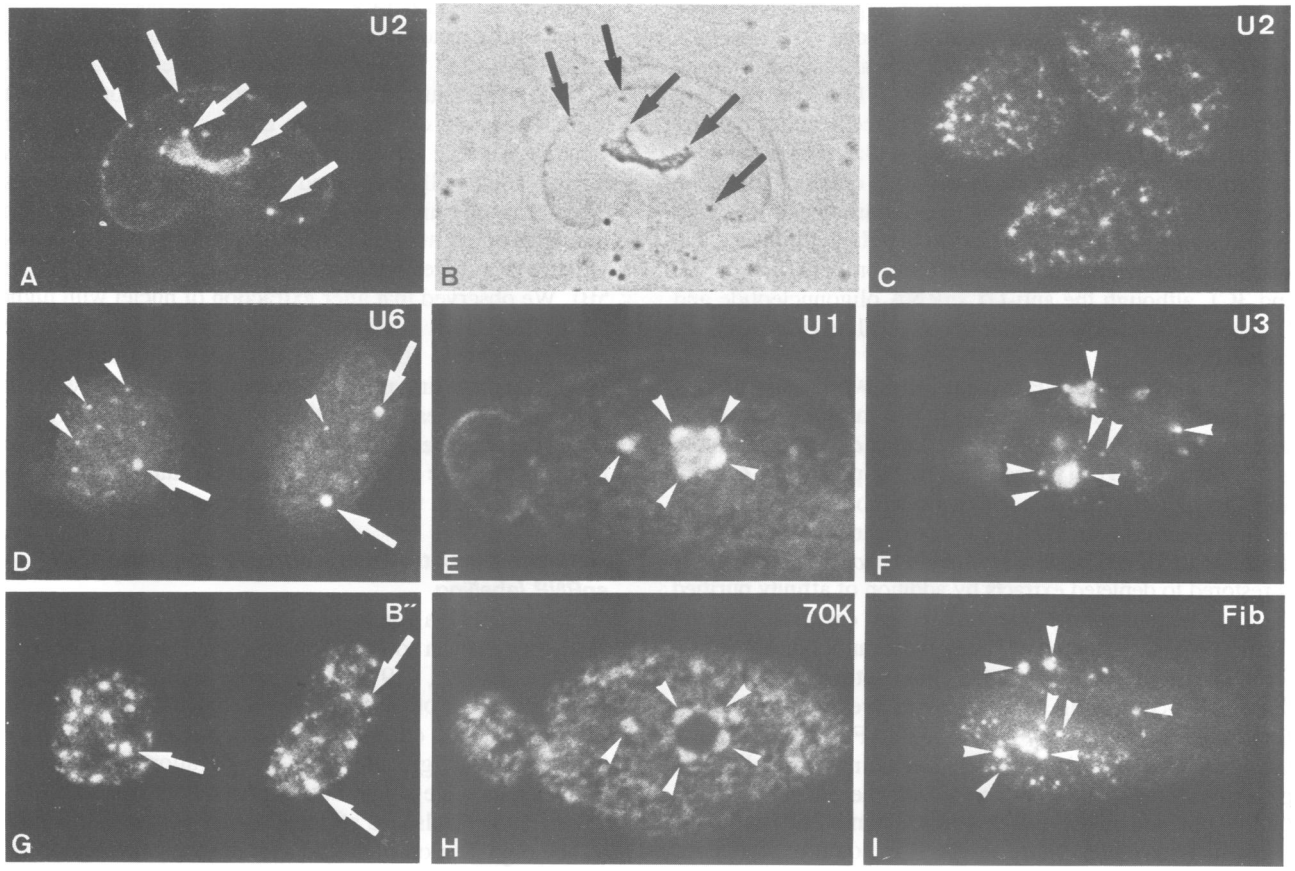

Fig. 5 . Labelling with the anti-U2 snRNA oligonucleotide probe reveals bright spots on nuclear matrices (**A**, arrows), which can be seen as dense structures by phase contrast microscopy (**B**, arrows). Exposure of detergent-extracted cells to 0.42 M KCl causes an apparent disruption of the spot pattern, as detected with an anti-U2 snRNA oligonucleotide probe (**C**). Actinomycin-treated cells were double-labelled with anti-U6 (**D**), U1 (**E**), U3 (**F**) snRNA oligonucleotide probes and anti-B″ (**G**), 70K (**H**), fibrillarin (**I**) antibodies. The anti-U6 snRNA probe labels spots with apparent variable sizes (D, arrows and arrowheads). Arrows point to co-localization of antisense RNA probe and antibody labelling (D, E, F and G, H, I). Magnification: (A–D, F, G and I) 1200×; (E and H) 1900×.

(Figure 5F), demonstrating that U3 snRNA remains associated with fibrillarin upon nucleolar disruption. A faint diffuse staining of the nucleolar remnants seen only with the antisense probe probably reflects a low level of nonspecific binding to pre-rRNA as previously discussed.

snRNP-containing foci are commonly found in mammalian cells

To examine whether the bright nuclear foci labelled by anti-snRNA oligonucleotide probes in HeLa cells are also present in other mammalian cells, five additional cell lines of both rodent and primate origin were tested (Figure 6A–E). In each case the anti-U2 snRNA oligonucleotide probe brightly labelled nuclear spots, akin to the foci seen in HeLa cells. A similar pattern was also seen in nuclei isolated from HeLa S3 cells (Figure 6F).

Discussion

We have identified foci in the nuclei of mammalian cells which contain the spliceosomal snRNPs together with the non-snRNP splicing factor U2AF. Detection of these structures has been greatly facilitated by *in situ* labelling using biotinylated 2′-OMe oligoribonucleotides specific for individual snRNAs. These probes bind selectively to targeted RNAs *in vitro* (Barabino *et al.*, 1989; Blencowe *et al.*, 1989; Lamond *et al.*, 1989; cf. Figure 1). This specificity is also apparent *in vivo* from the exclusive labelling of nucleoli by the anti-U3 snRNA oligonucleotide compared with the exclusively nucleoplasmic labelling by all the anti-spliceosomal snRNA probes. Four of the spliceosomal snRNA probes, i.e. those specific for U2, U4, U5 and U6 snRNAs, predominantly label the foci (Figure 2). As the mean number of foci per nucleus detected with each of these four probes is the same and does not increase when three probes are used for labelling simultaneously (Table I), we believe that these snRNAs are present together in the same structures. While formal proof of this awaits the development of a method for independently labelling separate oligonucleotides, snRNP co-localization is strongly supported by the observation that an anti-B″ antibody also labels the same mean number of bright spots per nucleus and co-localizes in these foci with each of the U2, U4, U5 and U6-specific antisense probes. In contrast with the other spliceosomal snRNAs, the antisense probe specific for U1 snRNA shows a widespread nucleoplasmic staining in addition to labelling foci. The anti-U2AF antibody similarly shows widespread nucleoplasmic labelling as well as staining the foci. Therefore, the components of the nuclear pre-mRNA splicing machinery detected by these probes are organized into two categories, i.e. those predominantly in foci (U2,U5 and U4/U6 snRNPs) and those widely distributed throughout the nucleoplasm as well as being in foci (U1 snRNP and U2AF).

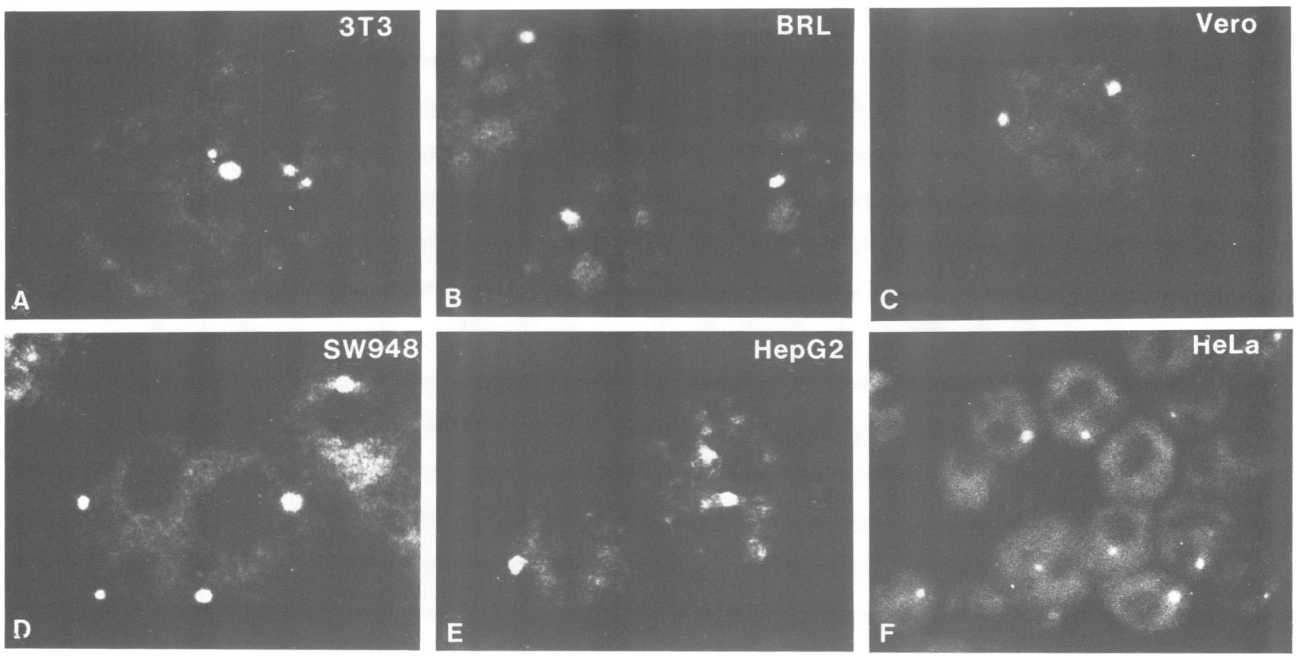

Fig. 6. Discrete nucleoplasmic spots labelled by the anti-U2 snRNA 2′-OMe oligonucleotide probe are observed on different cell lines, as revealed by confocal fluorescence microscopy: (**A**) 3T3, mouse fibroblasts; (**B**) BRL, Buffalo rat liver cells; (**C**) Vero, African green monkey kidney cells; (**D**) SW948, human colon adenocarcinoma cells; (**E**) HepG2, human hepatocellular carcinoma cells. Magnification: 2800×. Identical spots are observed on isolated nuclei from HeLa S3 cells (conventional fluorescence microscopy). Magnification: 1200×. Note that due to differences in the shape and size of the different cell lines it is not always possible to resolve the total number of foci in a single plane of focus.

The nuclear snRNP distribution in mammalian cells inferred from the labelling of snRNAs by oligonucleotide probes differs from that previously inferred from the labelling pattern of anti-snRNP protein antibodies (Spector *et al.*, 1984, 1990; Smith *et al.*, 1985; Nymann *et al.*, 1986). These studies reported a widespread punctate or speckled staining pattern for snRNP proteins, predominantly clustered into nonchromatin regions and possibly forming an interconnected nuclear reticulum. As each type of probe used to detect snRNP recognizes either snRNA or snRNP proteins, but not snRNP complexes *per se*, the reliable identification of assembled snRNPs requires the demonstration that both RNA and protein components are co-localized. The speckled nucleoplasmic labelling pattern reported using the B″-specific antibody has been interpreted as indicating a widespread distribution of U2 snRNP in the nucleoplasm (Habets *et al.*, 1989; Spector, 1990). In support of this interpretation it has been shown that in purified snRNP preparations, the B″ antigen is specifically associated with U2 snRNA and that the anti-B″ antibody selectively immunoprecipitates U2 snRNP from HeLa cell nuclear extracts (Habets *et al.*, 1989). We observe a similar speckled, nucleoplasmic staining with this anti-B″ antibody but in addition identify bright foci within this labelling pattern. These foci represent a distinct subset of the anti-B″ staining pattern which co-localizes with the foci labelled by anti-snRNA oligonucleotides. Furthermore, RNase treatment of cells abolishes labelling of foci, but not the additional nucleoplasmic speckles, by the anti-B″ antibody. Thus, using the criteria of co-localization, only the foci are shown to contain U2 snRNP. It is unclear whether the additional, speckled structures stained by the antibodies correspond to U2 snRNP particles that are inaccessible to detection by the 2′-OMe RNA antisense probes. However, our previous *in vitro* studies have shown that the anti-U2 snRNA probe can efficiently bind both free U2 snRNP and functional U2 snRNP that is present in pre- and post-splicing complexes (Barabino *et al.*, 1989; Lamond *et al.*, 1989). An alternative possibility is that the additional speckled staining represents either free B″ antigen or non snRNP-specific antibody binding. Although antibodies against m3G-cap and other snRNP-associated proteins show a similar speckled labelling of nuclei, an apparently identical pattern is also obtained using antibodies against nuclear antigens as diverse as oncogene proteins (Spector *et al.*, 1987), glutathione S-transferase (Bennett and Yeoman, 1985) and nuclear matrix components (Smith *et al.*, 1985) which have no known involvement in the pre-mRNA splicing process. A definitive identification of the presence of snRNP particles within these speckled staining regions therefore requires further analysis.

In the case of U1 snRNP, we have shown here that the staining pattern is dependent on the method of fixation (Figure 3). The widespread nucleoplasmic staining we observe using both anti-U1 oligonucleotide probes and U1-specific antibodies in Triton pre-extracted cells can be changed to the previously reported speckled or punctate pattern by fixing the cells instead in methanol−acetone. In addition, the co-localization of anti-m3G-cap and anti-snRNP protein antibody staining is also shown here to be dependent on the method of fixation. Therefore, we suggest that until further information is available, detailed inferences about snRNP distribution based on these staining patterns should be viewed with caution. An analysis of the snRNP distribution in unfixed cells is clearly needed to clarify the effect of different fixation methods. It should be emphasized, however, that the identification of nuclear foci containing spliceosomal components is not changed by the use of

different fixation methods. Rather, it is the appearance of the additional widespread nucleoplasmic staining pattern which is fixation method-dependent.

The snRNP-containing foci in mammalian nuclei may be related to the large nuclear 'spheres', which are prominently visible in the oocytes of amphibia and invertebrates (reviewed by Callan, 1986). These oocyte 'sphere organelles' have recently been shown to contain snRNPs by staining with anti-Sm and anti-m3G-cap antibodies (Gall and Callan, 1989). Future functional characterization of these snRNP-containing structures should help to determine whether the mammalian and amphibian structures are equivalent.

The co-localization of snRNAs, snRNP antigens and m3G-cap structures in foci indicates that mature snRNP particles are present, rather than nascent snRNA transcripts. Cap trimethylation and assembly of most snRNP proteins have been shown to take place in the cytoplasm prior to re-import of assembled snRNPs into the nucleus (reviewed by Mattaj, 1988; Zieve and Sauterer, 1990). The foci are therefore unlikely to be involved in the maturation of nascent snRNAs. In addition, the presence of U2AF, a non-snRNP splicing factor (see accompanying paper by Zamore and Green), also suggests that the foci are not likely to be some form of snRNP storage centres. It is possible that these structures are involved in the pre-assembly of mammalian snRNPs into spliceosome subunits, as proposed by Gall and Callan (1989), for the oocyte sphere organelles. If this is the case then the presence of U2AF in the foci implies that *in vivo* it is pre-assembled together with snRNPs prior to binding to pre-mRNA, in contrast to the snRNP-independent binding of U2AF to intron sequences observed *in vitro*. While a role for the foci in spliceosome pre-assembly is certainly possible, it is also plausible that they may instead play a more direct role in the pre-mRNA splicing reaction. The presence in foci of each of the spliceosomal snRNPs together with U2AF is equally consistent with either splicing itself occurring in the foci, or with their being a site for metabolism of excised introns and recycling of snRNPs after splicing has taken place. *In vitro* studies have shown that U2, U4, U5 and U6 snRNPs remain associated with the excised intron after completion of the splicing reaction (Konarska and Sharp, 1987).

It may be significant that the two components of the splicing machinery that show a widespread nuclear distribution, i.e. U1 snRNP and U2AF, correspond to the first components shown to bind to the 5′ and 3′ splice site regions respectively during spliceosome assembly. If the foci are really centres where splicing occurs, this observation suggests the possibility that newly synthesized transcripts could form a complex with U1 snRNP, U2AF and possibly other unidentified factors, which is then transported to the snRNP-containing foci for splicing. In support of this model, previous data based on *in situ* labelling experiments show an association of U1 snRNP with hnRNA at sites of active gene transcription (Sass and Pederson, 1984; Fakan *et al.*, 1986). What is less clear is whether other snRNPs in addition to U1 are also bound to nascent transcripts. The *in situ* labelling pattern reported for the hnRNP C proteins in HeLa cells (Choi and Dreyfuss, 1984) is also strikingly similar to that shown here for U1 snRNP in HeLa cells prepared by Triton pre-extraction. It will now be important to analyse whether any of the other recently reported mammalian splicing factors (Krämer, 1988; Fu and Maniatis, 1990; Krainer *et al.*, 1990), co-localize with either hnRNA transcripts or snRNP-containing foci.

Experiments are presently under way to test the different models discussed above and to investigate further the functional role of the nuclear foci in pre-mRNA processing.

Table II. Sequence of oligonucleotides

Oligonucleotide (2′-OMe RNA)	Sequence (5′−3′)	Length	Site of biotinylation
U1	CCUICCAIIUAAIUAU	16	3′
U2	IAACAIAUACUACACUU	17	3′
U3	UUUCIIUICUC	11	3′
U4	UACUICCACUICICAAAICU	20	5′
U5	UAGUAAAAGGCG	12	5′
U6	AUIIAACICUUCACIAAUUU	20	5′
ADENO	IACCAIAUIIACICIICC	18	5′

All oligonucleotides shown are made of 2′-OMe RNA except for the U5 probe which is made of 2′-OAllyl RNA. For both 5′ and 3′ biotinylation the biotin residues are linked via flexible alkyl spacer arms to additional, non-base-pairing deoxycytidine residues.

Materials and methods

Oligonucleotides and antisense affinity selection

Biotinylated oligonucleotides were synthesized as described by Sproat *et al.* (1989). All oligonucleotides except for the anti-U5 snRNA probe were made of 2′-OMe RNA. The U5 probe was made of 2′-OAllyl RNA as described by Iribarren *et al.* (1990). Sequences of the oligonucleotides and positions of biotin residues are listed in Table II. Each probe had four tandem biotin residues at either the 5′ or 3′ terminus. HeLa cell nuclear extracts were prepared as described by Barabino *et al.* (1990). snRNA affinity selection experiments and analysis of selected RNA species by Northern hybridization were done as described by Blencowe *et al.* (1989). The spliceosomal snRNAs (U1, U2, U4, U5 and U6) were detected using uniformly labelled riboprobes and the nucleolar snRNAs (U3 and U8), using 5′ end-labelled oligonucleotide probes.

Cell culture

HeLa cells, obtained from the laboratory of T.Kreis (EMBL), were grown on glass coverslips in Minimum Essential Medium supplemented with 1% glutamine, 10% fetal calf serum and antibiotics (Gibco). Cells were used after reaching a confluency of ~50%. For actinomycin treatment, cells were placed in fresh medium containing 5 μg/ml actinomycin D (Sigma) and grown for another 2 h before fixation.

Cell fractionation

Nuclear matrices were prepared following the procedure of Fey *et al.* (1986) on HeLa cells grown on coverslips. Matrices were obtained by digestion of chromatin with 100 μg/ml RNase-free DNase I (Boehringer) in the presence of RNasin (Promega), and extraction with 0.25 M ammonium sulphate.

For RNase digestion, HeLa cells grown on coverslips were incubated with buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) for 10 min on ice, extracted with 0.5% Triton X-100 in the same buffer for 3 min on ice and incubated with 25 μg/ml RNase A (Sigma) in the same buffer for 20 min at room temperature. 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma) was added in all steps.

Nuclei were isolated from HeLa S3 cells grown in suspension, as described (Dignam *et al.*, 1983) and attached to poly-L-lysine coated coverslips.

Extracted cells and isolated nuclei were fixed in paraformaldehyde and processed for *in situ* hybridization.

Fixation/permeabilization protocols

Cells on coverslips were rinsed twice with phosphate-buffered saline (PBS) and extracted with 0.5% Triton X-100 in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, 3 mM $MgCl_2$) (Fey *et al.*, 1986), containing 1 mM PMSF, for 3 min on ice. The cells were then fixed in 3.7% paraformaldehyde in the same buffer for 10 min at room temperature and washed in PBS. The coverslips were either used immediately for hybridization or stored in PBS at 4°C for up to 1 week. The following alternative methods of fixation/permeabilization were tested: (i) direct fixation

in 8% paraformaldehyde, 250 mM HEPES, pH 7.4, for 10 min and subsequent permeabilization in cold (−20°C) methanol for 10 min; (ii) fixation/permeabilization in cold methanol, 10 min, and fixation in 8% paraformaldehyde, 10 min; (iii) fixation/permeabilization in cold methanol and thereafter acetone (3 × 5 s dipping). Cells were washed in PBS before proceeding to hybridization or immunofluorescence.

In situ hybridization

Fixed cells were rinsed in 6 × SSPE (Maniatis *et al.*, 1982) and incubated for 5−15 min in either yeast or *Escherichia coli* tRNA (0.5 μg/μl in 6 × SSPE, 5 × Denhardt's solution). An equal volume of oligonucleotide probe diluted in 6 × SSPE, 5 × Denhardt's solution, was then added to the coverslips. The final concentration of biotinylated probe was 1−5 pM/μl and incubation times ranged from 5 min to 1 h in a humidified chamber at room temperature. Samples were extensively washed with gentle shaking in 6 × SSPE, at room temperature, and subsequently rinsed in 20 mM HEPES, pH 7.9, 0.15 M KCl, 0.05% Tween 20 (avidin wash buffer). Hybridization sites were detected using avidin conjugated to either fluorescein or Texas Red (E-Y Labs, San Mateo, CA). Samples were incubated in 2 μg/ml of avidin in freshly prepared 20 mM HEPES, 0.25 M KCl, 0.5 mM DTT, 1% BSA, for 5 min at room temperature. Longer incubation times were generally not necessary and in most cases resulted in an increase of unspecific staining. After washing in avidin wash buffer, the samples were either immunolabelled and mounted or directly mounted in Mowiol containing 100 mg/ml DABCO [1,4-diazabicyclo (2,2,2)octane, Sigma] as an antifading agent.

Immunofluorescence

RNPs were labelled with human autoimmune Sm and anti-(U1)RNP sera; monoclonal antibodies anti-Sm, Y12 (Pettersson *et al.*, 1984; Gerke and Steitz, 1986), anti-70K protein, 70K (Billings *et al.*, 1982) and anti-B″ protein, 4G3 (Habets *et al.*, 1989); polyclonal and monoclonal anti-2,2,7-trimethylguanosine antibodies (Reuter *et al.*, 1984); affinity purified rabbit anti-peptide antibody raised against human fibrillarin (Hurt *et al.*, 1990) and a purified antibody against a peptide from the 65 kd subunit of U2AF (Zamore and Green, 1991).

Directly after fixation or following hybridization, samples were rinsed in PBS with 0.05% Tween 20 (PBS−Tween) and incubated with the first antibodies diluted in the same buffer for 1 h. After extensive washing in PBS−Tween, antibody binding sites were revealed by goat anti-mouse, anti-rabbit or anti-human IgGs labelled with fluorescein, rhodamine or Texas Red.

Fluorescence microscopy

Samples were visualized using 63× or 100× objectives on a Zeiss Axiophot microscope equipped with epifluorescence. Photographs were taken on T max 400 film (Kodak).

Confocal fluorescence microscopy

The modular confocal microscope, developed and constructed at EMBL, was used. Excitation wavelengths of 488 nm (for single-labelled fluorescein fluorescence) or 514.5 nm (for double-labelled fluorescein and rhodamine or Texas Red fluorescence) were selected from an Argon-ion laser. Confocal image series of cells were recorded at 0.4 μm per vertical step. Projections of each series were calculated. For double-labelling experiments both fluorochromes were simultaneously recorded by two detectors. Pseudo-coloured images of both signals were generated and superimposed. Images were photographed on Fujichrome 100 or Kodak T max 100 film, using a Polaroid Freeze Frame Recorder.

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