Cell Biology. In the article "U1 and U2 small nuclear RNAs are present in nuclear speckles" by Sui Huang and David L. Spector, which appeared in number 1, January 1992, of *Proc. Natl. Acad. Sci. USA* (**89**, 305–308), Figs. 1 and 2 were poorly reproduced. The figures and their legends are shown here.

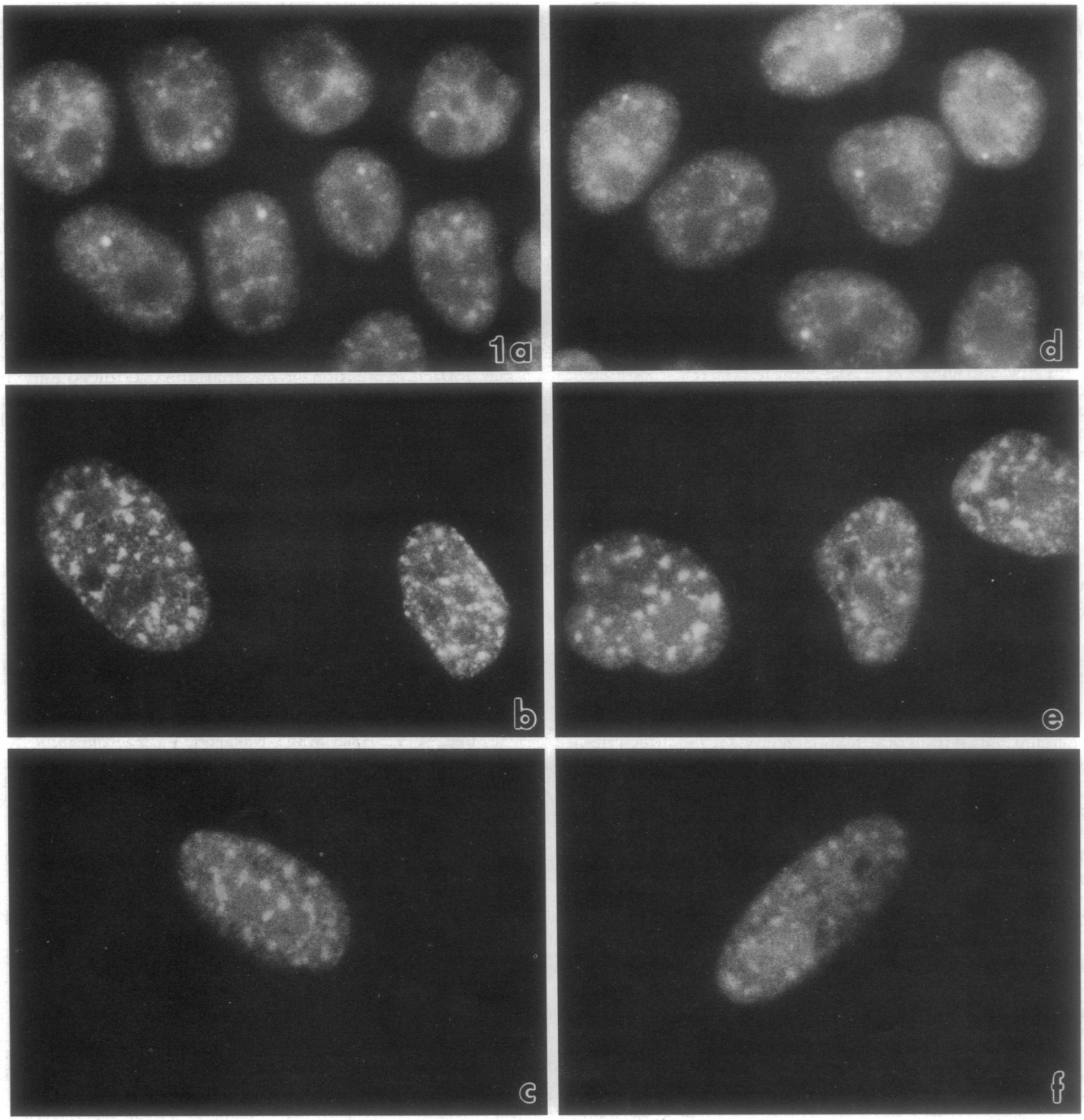

FIG. 1. Distribution of U1 (*a–c*) and U2 (*d–f*) snRNAs in interphase nuclei of HeLa (*a* and *d*), Detroit 551 (*b* and *e*) and WI-38 (*c* and *f*) cells. These snRNAs are localized in a speckled distribution pattern in all cells examined. In addition, intensely stained foci are also observed in HeLa cells (*a* and *d*). Foci were not observed in Detroit 551 (*b* and *e*) or WI-38 (*c* and *f*) cells. (×850.)

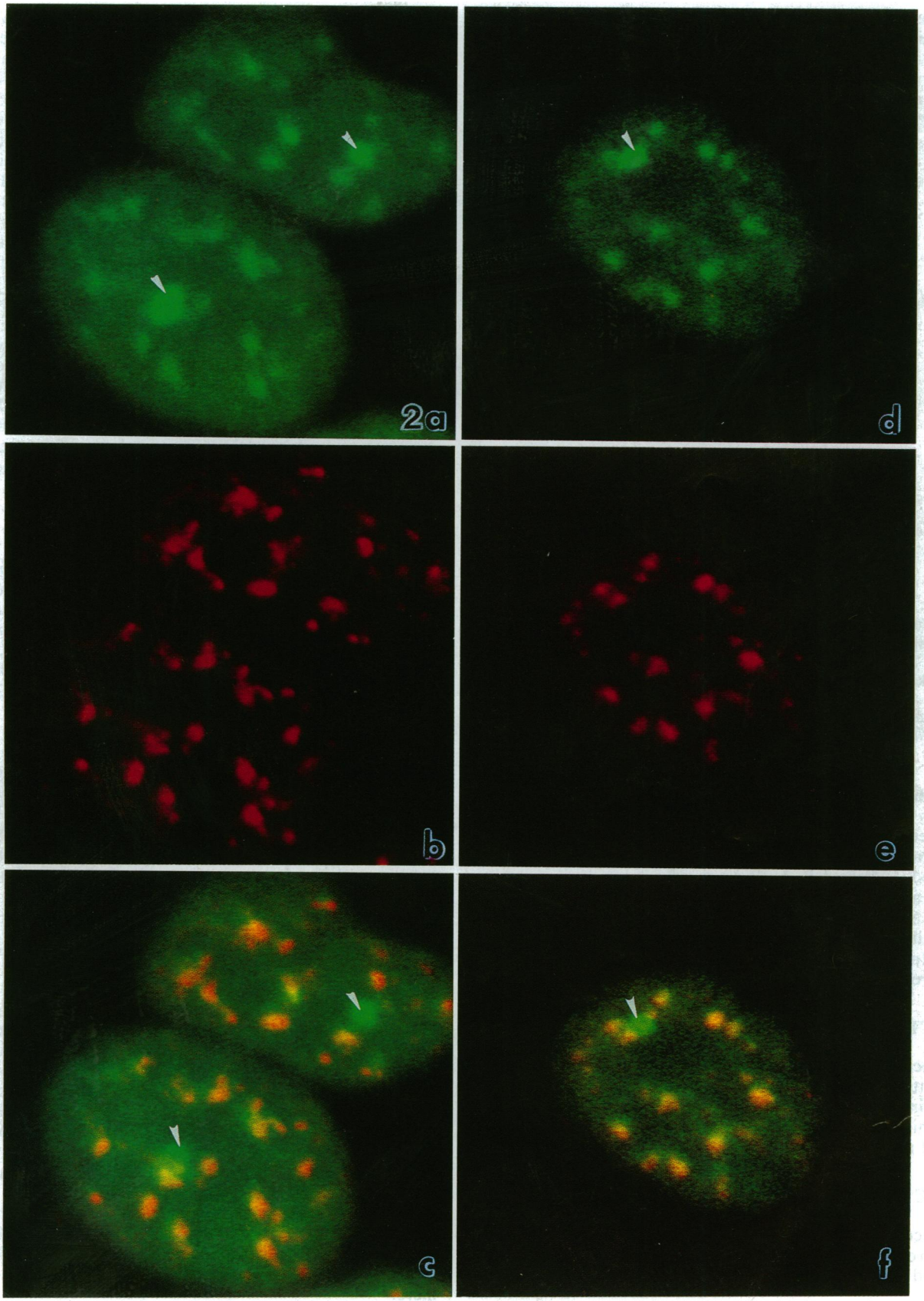

FIG. 2. Colocalization of U1 and U2 snRNAs with the essential non-snRNP splicing factor SC-35. HeLa cells were hybridized with oligonucleotide probes complementary to U1 (*a*) or U2 (*d*) snRNA and were then immunolabeled with anti-SC-35 monoclonal antibody (*b* and *e*). Oligonucleotide probes (11) were detected with fluorescein-conjugated avidin DCS and anti-SC-35 antibodies were detected with Texas Red-conjugated secondary antibodies. In superimposed images, the colocalization of fluorescein-labeled and Texas Red-labeled structures appears yellow (*c* and *f*). Both U1 (*c*) and U2 (*f*) snRNAs colocalize with SC-35 in a speckled nuclear distribution (yellow regions). In addition, U1 and U2 snRNAs are also present in foci (arrowheads in *a* and *d*) that do not colocalize with SC-35 (arrowheads in *c* and *f*). (×2950.)

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U1 and U2 small nuclear RNAs are present in nuclear speckles

(RNA processing/nuclear organization)

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ABSTRACT **The localization of U1 and U2 small nuclear RNAs (snRNAs) has been examined by *in situ* hybridization using 2′-*O*-alkyl oligonucleotide probes. We have found that these snRNAs, which are essential for pre-mRNA splicing, localize in a speckled distribution, in addition to being present in three or four foci, in HeLa cell nuclei. However, in cells of defined passage, such as Detroit 551 and WI-38 fibroblasts, these snRNAs are concentrated in nuclear speckles, and foci are not observed. The speckled distribution of U1 and U2 snRNAs is coincident with the speckled regions enriched in small nuclear ribonucleoprotein particle (snRNP) proteins and the essential non-snRNP splicing factor SC-35. The localization of these key components of the pre-mRNA splicing machinery to speckled nuclear regions suggests that these regions may be involved in pre-mRNA splicing.**

The small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U5 have been shown to function in the processing of pre-mRNA (for review see refs. 1 and 2). Localization studies using snRNP protein-specific antibodies (3–9) or antibodies to the trimethylguanosine (m_3G) cap structure of snRNAs (ref. 10; D.L.S. and A. Krainer, unpublished work) have shown that these splicing components are concentrated in 20–50 speckled nuclear regions that form a three-dimensional latticework in the interphase nucleus (6). However, recent *in situ* hybridization studies using 2′-*O*-alkyl antisense RNA oligonucleotide probes to various small nuclear RNAs (snRNAs) indicate that the localization of U2, U4, U5, and U6 snRNAs is restricted to three or four round foci and that U1 snRNA is diffusely distributed as well as being present in foci (11, 12). At least two possible explanations can be considered to explain the differences in snRNP-antigen and snRNA localization. First, snRNAs and associated proteins may localize to three or four foci and the other speckled nuclear regions may contain only snRNP proteins. However, this is unlikely since the localization of the m_3G cap structure suggests that at least one snRNA is present in the speckled regions (10). Alternatively, the snRNAs may be present in the speckled nuclear regions but may be less accessible to oligonucleotide probes. Since snRNAs are essential components of the pre-mRNA splicing machinery, it is important to determine their nuclear organization, which in turn indicates where splicing is likely to take place. To differentiate between the above possibilities, we have examined the localization of U1 and U2 snRNAs by using the oligonucleotide probes described by Carmo-Fonseca *et al.* (11, 12). We have found that both U1 and U2 snRNAs are concentrated in speckled nuclear regions, as well as foci, in HeLa cells; detection of snRNAs in the speckled regions requires hybridization with 2′-*O*-alkyl probes for longer periods of time than previously reported (11, 12). In addition, when these probes are hybridized to cells of defined passage (Detroit 551, WI-38), U1 and U2 snRNAs are concentrated in nuclear speckles, and foci are not observed. Furthermore, U1 and U2 snRNAs colocalize to the same speckled nuclear regions labeled with antibodies specific for snRNP antigens and the non-snRNP splicing factor SC-35.

METHODS

Cell Culture. HeLa cells were grown on glass coverslips in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum. Detroit 551 and WI-38 cells were grown in minimum essential medium (MEM) containing 10% fetal bovine serum.

***In Situ* Hybridization.** Cells were prepared for *in situ* hybridization according to Carmo-Fonseca *et al.* (11). Cells were permeabilized with 0.5% Triton X-100 in CSK buffer (0.1 M NaCl/0.3 M sucrose/10 mM Pipes, pH 6.8/3 mM $MgCl_2$) containing 1 mM phenylmethylsulfonyl fluoride on ice for 3 min. Cells were then fixed in freshly prepared 3.7% formaldehyde in CSK buffer for 10 min at 21°C and washed three times (10 min per wash) with phosphate-buffered saline (PBS). After rinsing in 6× standard saline/phosphate/EDTA (SSPE) (13), cells were prehybridized with 6× SSPE/5× Denhardt's solution containing *Escherichia coli* tRNA (0.5 μg/ml) for 15 min. Cells were subsequently hybridized in the same buffer with biotinylated 2′-*O*-alkyl antisense oligonucleotide probe (1.2 pmol/μl) to U1 or U2 snRNA for 16–24 hr at 21°C. Cells were washed three times (15 min each) in 6× SSPE while shaking and then rinsed with avidin wash buffer (0.02 M Hepes, pH 7.9/0.15 M KCl/0.05% Tween 20) for 15 min. Hybridization signal was detected by incubation with fluorescein-conjugated avidin DCS (Vector Laboratories) at 2 μg/ml in avidin wash buffer containing 1% bovine serum albumin for 30 min at 21°C. Cells were washed three times (15 min each) in avidin wash buffer and mounted in 90% glycerol/10% PBS containing *p*-phenylenediamine at 1 mg/ml. The final pH was adjusted to 8.0 with 0.5 M carbonate/bicarbonate buffer, pH 9.0. Cells were examined with a Nikon FXA epifluorescence microscope equipped with a ×60, 1.4-n.a. objective lens.

Immunolabeling and Confocal Microscopy. After detection of the hybridization signal by fluorescein-conjugated avidin, cells were washed three times (10 min each) in PBS containing 1% normal goat serum. Cells were then incubated with anti-SC-35 monoclonal antibody (14) at a dilution of 1:50 for 1 hr and rinsed three times (10 min each) in PBS containing 1% normal goat serum. Cells were then incubated with Texas Red-conjugated goat anti-mouse antibody at a dilution of 1:20 for 1 hr followed by three washes (10 min each) in PBS. Optical sections of 0.5 μm were obtained using a Zeiss confocal laser scanning microscope equipped with a ×63, 1.4-n.a. objective lens, an argon ion laser (488 nm, excites fluorescein) and a He/Ne laser (543 nm, excites Texas Red). Individual fluorescein and Texas Red signals from the same

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Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle.

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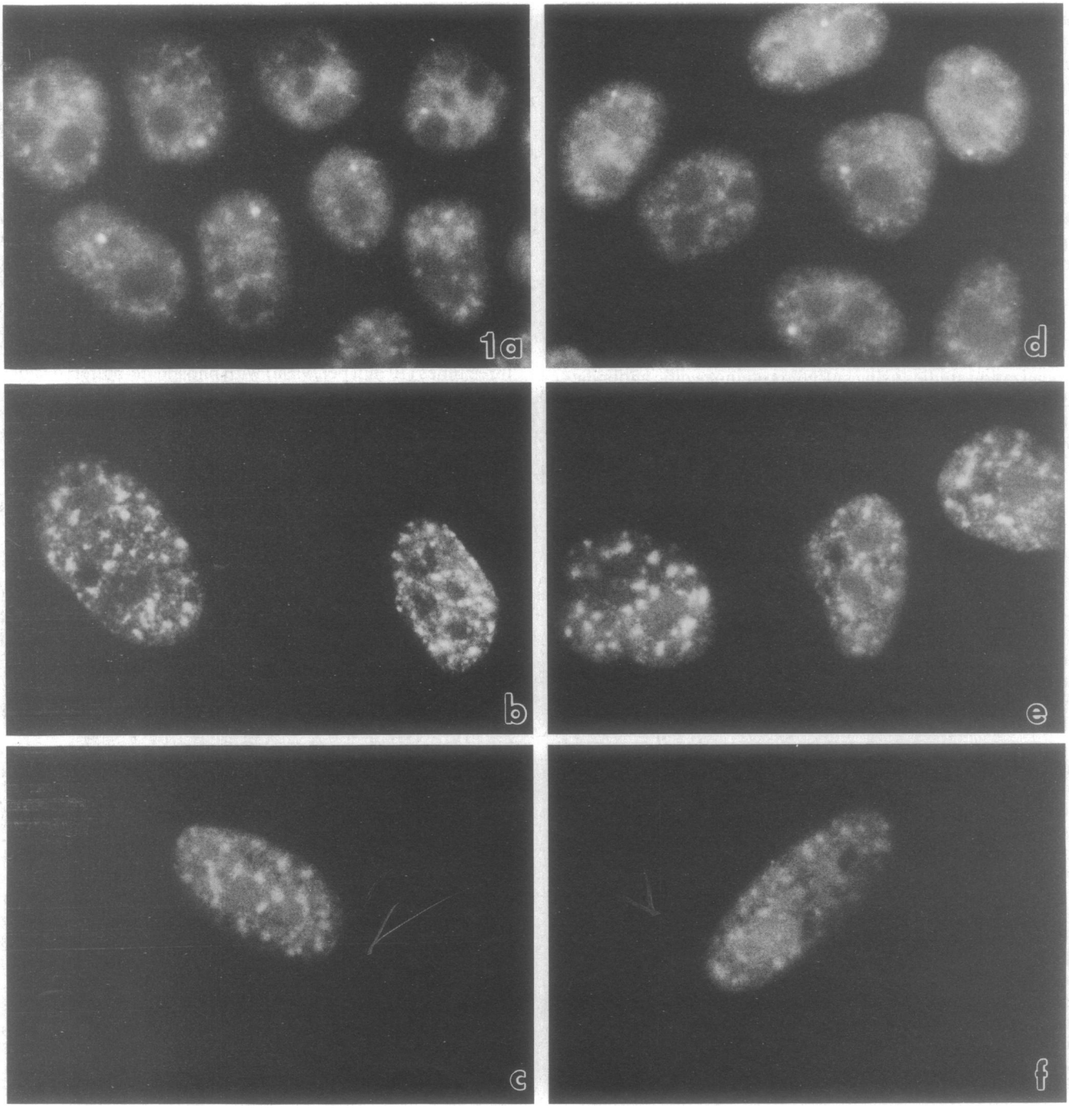
optical section were pseudocolored and superimposed using a Zeiss computer package (version 2.03).

RESULTS AND DISCUSSION

In previous studies, hybridization of cells with 2'-*O*-alkyl antisense oligonucleotide probes to various snRNA species for 5 min to 1 hr showed U2, U4, U5, and U6 snRNAs to be concentrated in three or four foci, whereas U1 snRNA was diffusely distributed in the nucleus in addition to being present in foci (11, 12). To determine whether the lack of snRNA localization in snRNP antigen-enriched nuclear speckles was a consequence of probe accessibility, we extended the hybridization times with these same probes (11, 12) to 16–24 hr. The specificity of the oligonucleotide probes has been demonstrated (11). With the extended hybridization time, both U1 and U2 snRNAs were shown to be concentrated in a speckled nuclear distribution pattern in addition to being present in three or four foci in HeLa cells (Fig. 1 *a* and *d*). Depending upon the cell type examined, varying degrees of diffuse staining were also observed (Fig. 1 *a–c*). The concentration of U1 and U2 snRNAs in speckled nuclear regions suggests that these RNAs may be tightly associated with snRNP proteins and/or other spliceosomal components, thereby limiting the accessibility of them to the antisense probes during the short hybridization time used in other studies (11, 12). It has been reported that spliceosomes are composed of at least 50 proteins, suggesting that snRNPs may



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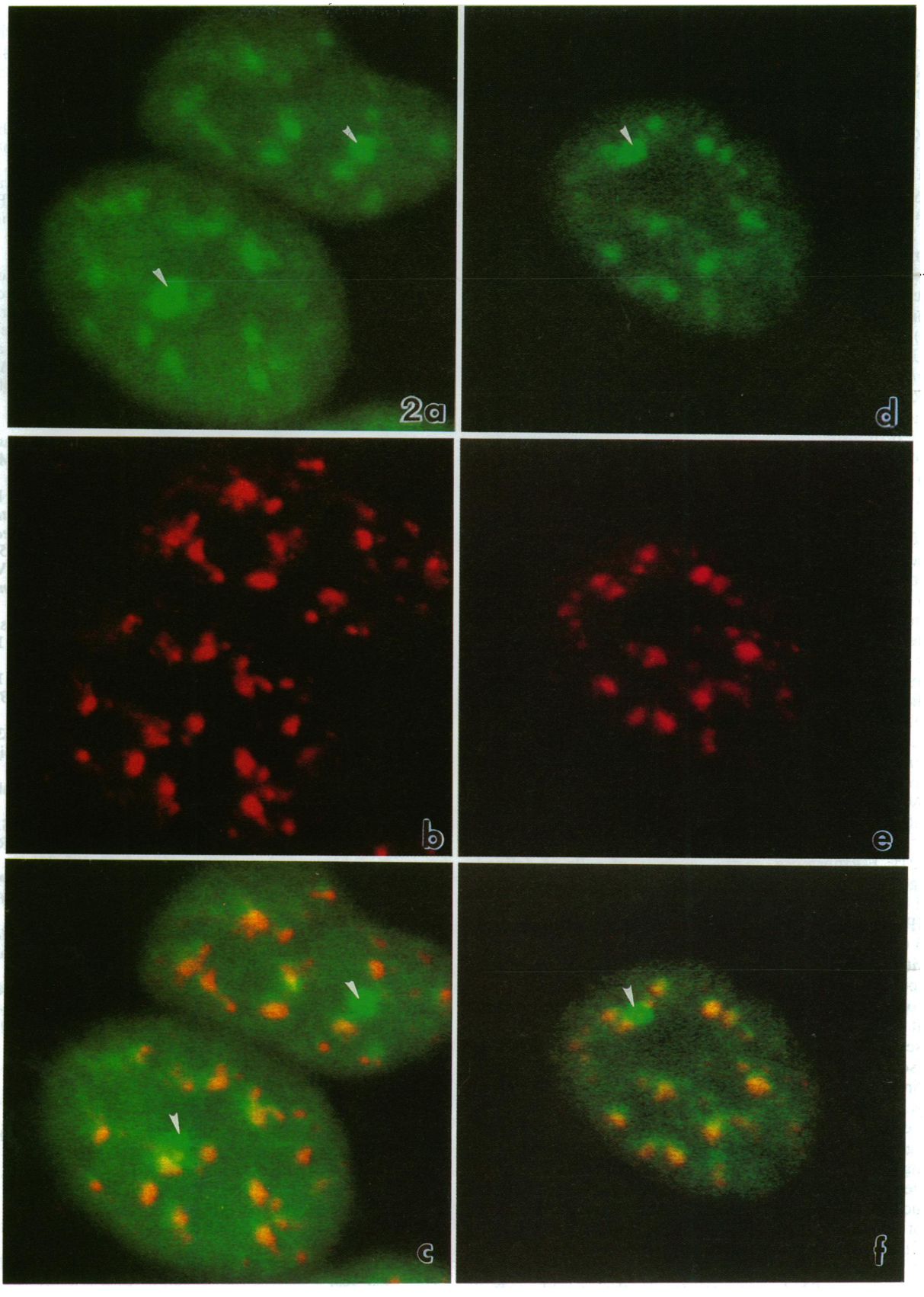

FIG. 2. Colocalization of U1 and U2 snRNAs with the essential non-snRNP splicing factor SC-35. HeLa cells were hybridized with oligonucleotide probes complementary to U1 (*a*) or U2 (*d*) snRNA and were then immunolabeled with anti-SC-35 monoclonal antibody (*b* and *e*). Oligonucleotide probes (11) were detected with fluorescein-conjugated avidin DCS and anti-SC-35 antibodies were detected with Texas Red-conjugated secondary antibodies. In superimposed images, the colocalization of fluorescein-labeled and Texas Red-labeled structures appears yellow (*c* and *f*). Both U1 (*c*) and U2 (*f*) snRNAs colocalize with SC-35 in a speckled nuclear distribution (yellow regions). In addition, U1 and U2 snRNAs are also present in foci (arrowheads in *a* and *d*) that do not colocalize with SC-35 (arrowheads in *c* and *f*). (×2950.)

be components of very complex macromolecular structures (15). However, when cells are hybridized for a longer period of time, the accessibility of the oligonucleotide probes to the snRNAs may be increased, so that the speckled distribution pattern is revealed in addition to the foci. Cells pretreated with RNase A prior to *in situ* hybridization did not exhibit speckles or foci (data not shown).

To demonstrate that this speckled organization of snRNAs is not unique to HeLa cells, we examined two human diploid cell types. The speckled distribution of U1 and U2 snRNAs was also observed in Detroit 551 (Fig. 1 *b* and *e*) and WI-38 (Fig. 1 *c* and *f*) cells, which have a defined passage number. In contrast to our observations in HeLa cells, these cells did not exhibit foci. The lack of foci staining is consistent with studies using antibody probes, which have shown that the presence of snRNPs as foci is cell type-specific (unpublished work). These results demonstrate that the organization of U1 and U2 snRNAs into a speckled pattern is common in many mammalian cell types.

snRNP antigens and a non-snRNP splicing factor, SC-35, colocalize to a speckled pattern in the nucleus (14, 16). This speckled distribution pattern observed by fluorescence microscopy represents nuclear substructures termed interchromatin granules and perichromatin fibrils (16). Perichromatin fibrils are thought to represent RNA transcripts (17, 18), while interchromatin granules may represent RNP storage (19, 20) and/or assembly sites (16). To address the question of whether the U1 and U2 snRNAs are also part of the same staining regions, we compared the localization of U1 and U2 snRNAs with the localization of snRNP antigens (data not shown) and SC-35 (Fig. 2) in HeLa cells. As shown in double-label experiments (Fig. 2) the speckled distribution of U1 or U2 snRNAs is coincident with that of SC-35, demonstrating the colocalization of snRNAs with nuclear regions enriched in snRNP antigens and a non-snRNP splicing factor. This observation is consistent with the demonstration that the m_3G cap structure of the snRNAs is also localized to these same nuclear regions (10). However, the intensely stained foci do not overlap with the localization of SC-35 (Fig. 2 *c* and *f* arrowheads) (12). These foci have recently been identified as coiled bodies (D.L.S., G. Lark, and S. Huang, unpublished work), RNP-containing structures of unknown function (21). Furthermore, in the majority of cells of defined passage number, which do not exhibit snRNP-containing coiled bodies, there is a complete colocalization of snRNAs, snRNP proteins, and the essential non-snRNP splicing factor SC-35 (data not shown). The absence of coiled bodies in the majority of cells of defined passage (unpublished work) has been confirmed using a coiled-body-specific antibody (21). The colocalization of snRNAs with snRNP proteins is consistent with studies in amphibian germinal vesicles (22).

Based on this study and previous studies (23, 24), snRNPs are associated with at least three different nuclear structures: perichromatin fibrils, interchromatin granules, and coiled bodies. We have localized U1 and U2 snRNAs to the same speckled nuclear regions (perichromatin fibrils and interchromatin granules) where snRNP antigens (3–9) and the essential splicing factor SC-35 (14, 16) have been localized. These speckles interconnect to form a three-dimensional network in the nucleus (6, 16). Since snRNA-containing coiled bodies are not detectable in all cells of a given population, it is less likely that these nuclear inclusions are essential for pre-mRNA splicing. Wang *et al.* (25) have shown that an intron-containing pre-mRNA concentrates in the speckles after microinjection into the cell nucleus. We have reported (26) that serum-induced endogenous nascent c-*fos* RNA transcripts are closely associated with the snRNP-enriched nuclear speckles in NIH 3T3 cells. In addition, pulse labeling of cells with [^{3}H]uridine revealed incorporation of autoradiographic grains over perichromatin fibrils (17, 18), which are a component of the snRNP speckled network (16). These studies suggest that the speckled regions enriched in splicing components play a role in pre-mRNA splicing.

Note Added in Proof. Prehybridization of cells with unlabeled U2 oligonucleotide blocks the subsequent hybridization of biotinylated U2 2′-*O*-methyl oligonucleotide. Under these conditions, labeling of neither speckles nor coiled bodies was observed, indicating the specificity of the hybridization signal. We thank Albrecht Bindereif (Max Planck Institute, Berlin) for providing U2-specific oligonucleotides.

We thank Dr. Angus Lamond (European Molecular Biology Laboratory) for providing us with 2′-*O*-alkyl oligonucleotide probes and Drs. Xiang-Dong Fu and Tom Maniatis (Harvard University) for providing us with anti-SC-35 antibodies. We appreciate helpful comments from Joe Gall, Scott Henderson, Adrian Krainer, and Tom Maniatis. This study was supported by grants from the American Cancer Society (NP-619A) and the National Institutes of Health (GM42694 and 5P30 CA45508-03) to D.L.S.

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