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SUBCELLULAR LOCATION OF POLYPEPTIDES THAT REACT WITH ANTI-Sm AND ANTI-RNP ANTIBODIES

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SUMMARY: Whole nuclear and cytoplasmic fractions from HeLa cells were analyzed in protein gel blots probed with either monoclonal anti-Sm or polyclonal anti-(U1)RNP antibodies. The cells were fractionated by a nonaqueous procedure, to minimize proteolysis and artifactual leakage of nuclear components to the cytoplasmic fraction. Unexpectedly, more reactive proteins were detected in the nucleus than shown earlier in partially purified small nuclear ribonucleoprotein particles (snRNPs). In addition, reactive polypeptides were now found in the cytoplasm. These results are discussed in reference to the possibility that the nucleus and cytoplasm of adult somatic human cells may have a more complex than anticipated set of populations of polypeptides bearing Sm or RNP antigenic determinants, including some proteins that might not be in snRNP form.

U1, U2, U4, U5 and U6 snRNAs* are present in eukaryotic cells as constituents of snRNPs* (1). Each snRNA species appears to exist in a different snRNP, and the protein composition of the various snRNPs seems to be quite similar (1-4). U1 snRNP may have a function in the splicing of messenger RNA precursors (5-8). Anti-RNP antibodies react with U1 snRNP, while anti-Sm antibodies react with all five snRNP species (1). In the process of putting together clues about the function, metabolism and regulation of the cellular level of various snRNP species and their components, it would be useful to identify the proteins that bear RNP- and Sm-types of antigenic determinants, as well as the subcellular location and level of these polypeptides, and to determine whether they are in snRNP form or not. Protein gel blot analyses using anti-Sm and anti-(U1)RNP antibodies as probes have been done previously with

* Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; SDS, sodium dodecyl sulfate; PBS, 10 mM phosphate, pH 7.2/0.14 M NaCl; PBS-T, PBS/0.05% Triton X-100; Ig, immunoglobulin.

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either partially purified snRNPs or the supernatant from broken nuclei (2, 9, 10), but not with total nuclear or cytoplasmic fractions. Therefore, there was a possibility that some nuclear and/or cytoplasmic polypeptides carrying these antigenic determinants might not have been detected, and this was examined in the present report. In vivo, virtually all (if not all) molecules of U snRNAs are present in the nucleus (11) and in snRNP form (1,12). Therefore, we chose to fractionate HeLa cells by a nonaqueous procedure (13), to minimize artifactual leakage of snRNPs to the cytoplasmic fraction.

METHODS: Cell Radiolabeling. HeLa cell monolayers were labeled by incubation with [^{3}H]uridine (7 μCi/ml) for 9 h, or used without labeling. Nonaqueous Cell Fractionation. This procedure has been described (13). Briefly, cells were lyophilized, suspended at 4 x 10^7 cells/ml in hexylene glycol, and sonicated at -35°C. Nuclei were pelleted by centrifugation in propylene glycol for 90 min, 30,000 rpm at -40°C. The cytoplasmic material was then sedimented in a second centrifugation for 240 min, 30,000 rpm at -10°C. Gel Electrophoresis (14). Stacking and separating gels of 4.5% and 10% polyacrylamide, respectively, were used. Before electrophoresis, the samples were diluted in 0.125 M Tris-HCl, pH 6.8/5% 2-mercaptoethanol/3% SDS, and boiled for 5 min. Protein Gel Blot Analysis (15). Immunoblots were performed as described (16). After electroblotting to nitrocellulose, each sheet was treated with 0.25% gelatin in PBS, incubated with 1 mg of Ig in 10 ml of PBS-T for 2 h, washed, and then incubated with 10 μCi of ^{125}I-labeled protein A (New England Nuclear) in 35 ml of PBS-T for 1 h. Fluorography and Autoradiography. ^{3}H-labeled RNA was analyzed by fluorography (17) using sodium salicylate (18). Prefogged (19) X-ray film was used for fluorography and ^{125}I autoradiography. Exposures were done at -85°C. The ^{125}I-labeled blots were placed between two calcium tungstate intensifying screens. Preparation of Immunoglobulins. Human Ig was isolated from serum by DEAE cellulose chromatography followed by precipitation with 45% saturated ammonium sulfate. The specificity for U1 snRNP of our human polyclonal anti-RNP antibody preparation was shown earlier by affinity chromatography of extracts from [^{3}H]uridine-labeled cells (20). The hybridoma cell line producing monoclonal anti-Sm antibodies (21) was kindly supplied by Drs. E.A. Lerner and C. A. Janeway, Jr.. Monoclonal anti-Sm antibodies were prepared as described (21), except that AKD2F_1/J mice (Jackson Laboratory, Bar Harbor, Maine) were used for growth in the peritoneal cavity. Their anti-Sm specificity was confirmed by RNA analysis after immunoprecipitation of extracts from cells that had been radiolabeled in vivo (1) (not shown).

RESULTS: Two proteins, with approximate molecular weights of 28,000 and 16,000 (28K and 16K), have previously been detected by protein gel blot analysis of partially purified snRNPs or of the supernatant from broken nuclei, using monoclonal anti-Sm antibodies as probes (2). When we analyzed a total nuclear fraction by the immunoblot technique, the 28K protein was detected but comparable levels of the 16K protein were not seen (Fig. 1, lanes 1-3). In addition, some fainter bands were observed: ~74K; ~53K and ~48K. After

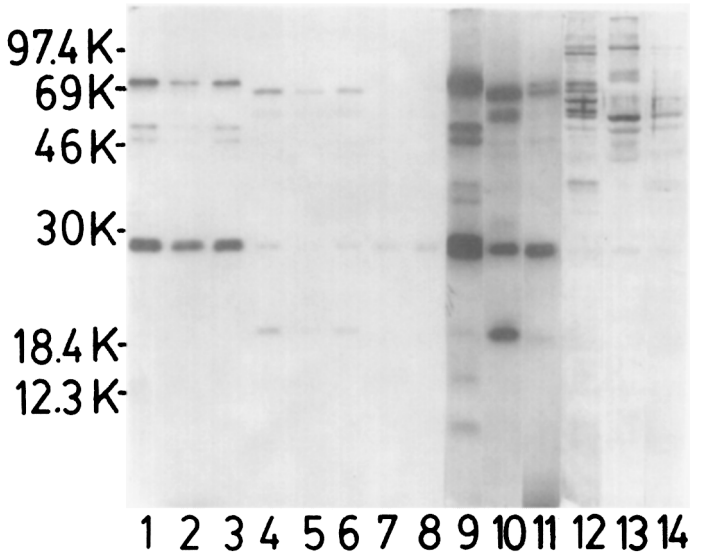

Fig. 1. Protein gel blot analysis using monoclonal anti-Sm antibodies (21) as the detection probe with ^{125}I-labeled protein A. Lanes 1-11 are autoradiographic images. They show 8 μl [1] and 3 μl [2] of the nuclear fraction, 8 μl [4] and 3 μl [5] of the cytoplasmic fraction, and 4 μl of unfractionated cells [7] from one nonaqueous cell fractionation experiment; 8 μl of the nuclear fraction [3], 8 μl of the cytoplasmic fraction [6], and 8 μl of unfractionated cells [8] from a second nonaqueous cell fractionation experiment. Lanes 1-8 are from a single X-ray film exposure of 1 h. Lanes 9,10, and 11 are an X-ray film exposure of 7 h and 15 min of lanes 1,4 and 8, respectively. The India ink-stained (22) proteins of blot lanes 1,4 and 7 are shown in 12,13 and 14 respectively. All the lanes in this figure are from one electrophoresis gel slab and one nitrocellulose blot. It is estimated that there were ~1.5-fold more cell equivalents in the nuclear lanes than in the corresponding cytoplasmic lanes [1 and 4, or 2 and 5] (lanes 1 and 2 in Fig. 2). This was calculated from densitometry of the fluorography bands of ^{3}H-labeled U1 and 4S RNA in nuclear, cytoplasmic and whole cell samples (Fig. 2, lanes 7-9).

longer X-ray film exposure (Fig. 1, lane 9), bands at ~39K, ~36K and three smaller ones were detected, including one at ~14.5K which might be the 16K protein previously reported. Analysis of the cytoplasmic fraction revealed some 28K protein and several polypeptides that were not seen in nuclei: ~69K; ~58K and ~20K (Fig. 1, lanes 4-6 and 10). The reproducibility of both the nuclear/cytoplasmic partitions and the patterns of proteins detected by immunoblots was very good, as seen in the two separate nonaqueous cell fractionation experiments shown (Fig.1).

Protein gel blot analysis of partially purified snRNPs or of the supernatant from broken nuclei revealed that various human anti-(U1)RNP antibodies reacted primarily with one or more of three proteins, that are U1 snRNP-spe-

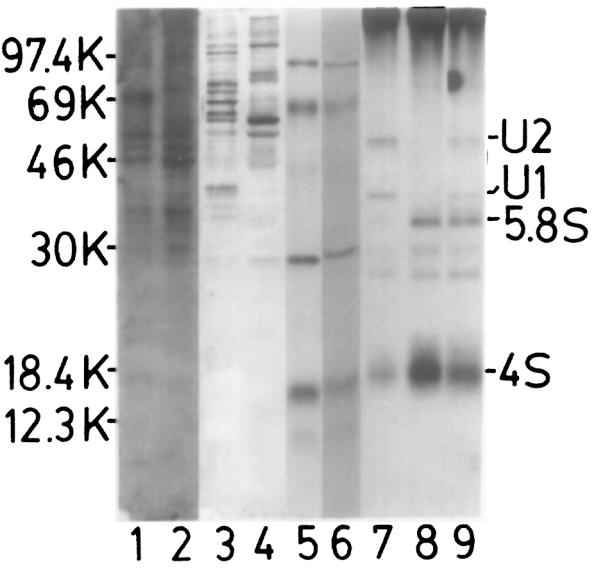

Fig. 2. Protein gel blot analysis using polyclonal anti-(U1)RNP antibodies as the detection probe. The lanes are blot autoradiography of 8 μl of the nuclear fraction [1] and 8 μl of the cytoplasmic fraction [2] from the first nonaqueous fractionation experiment shown in Fig. 1, after sequential incubations with anti-(U1)RNP antibodies and with ^{125}I-labeled protein A. Lanes 1 and 2 are from the same X-ray film exposure. The India ink-stained proteins of blot lanes 1 and 2 are shown in 3 and 4, respectively. A commercial mixture of ^{14}C-labeled proteins was loaded on several wells of the gel. One of the gel lanes was dried on paper, without blotting, and was placed on X-ray film [5]; another gel lane was electroblotted, and X-ray film was exposed to the blot [6]. Lanes 5 and 6 are included to show the similar blotting efficiency of polypeptides of various sizes. Therefore, care was not taken to display comparable X-ray film exposures of these two lanes or to match the magnifications of these two photographs with respect to the rest of the figure (that is why the migration of the proteins in 5 and 6 does not coincide with the molecular weight markings on the left side of the figure). The HeLa cells for the first nonaqueous fractionation mentioned in Fig. 1 had been labeled with [^{3}H]uridine (see Methods). Fluorography of the low molecular weight RNA pattern (three nonblotted gel lanes) is shown: 3 μl of the nuclear fraction [7]; 3 μl of the cytoplasmic fraction [8]; and 2 μl of unfractionated cells [9]. All the lanes in this figure are from a single gel slab.

cific: 22K; 33K and 68-70K (2,9,10). In a total nuclear fraction, our anti-(U1)RNP antibodies recognized only one of these proteins (~70K), plus other fainter bands (Fig. 2, lane 1). None of the three U1 snRNP-specific proteins could be detected in the cytoplasmic fraction; instead, a major band was seen (~45K), plus several other bands of lower intensity (Fig. 2, lane 2).

Two observations suggest that the antibody staining reactions were specific. First, the immunostained proteins were not among the most abundant proteins in the nucleus or cytoplasm, comparing the autoradiographs of the pro-

tein gel blots with the India ink-stained (22) protein patterns (Fig. 1, lanes 9-14; Fig. 2, lanes 1-4). Second, these polypeptides could not be seen in protein gel blots that were probed with nonimmune Ig (not shown). The immunoblots were done with an excess of antibodies and radiolabeled protein A. The nuclear and cytoplasmic proteins analyzed were the limiting factor in the assay, as shown by testing different amounts (Fig. 1, lanes 1, 2, 5 and 6). Therefore, the levels of polypeptides detected should reflect their actual concentrations.

DISCUSSION: The monoclonal anti-Sm antibodies reacted with more proteins from a total nuclear fraction (Fig. 1) than from partially purified snRNPs (2). Similarly, more polypeptides from a total nuclear fraction were recognized by our anti-(U1)RNP antibodies (Fig. 2) than of partially purified snRNPs or of the supernatant from broken nuclei probed with comparable antibody preparations (2,9,10). A possible explanation is that the additional proteins could be present in vivo in snRNP form but might easily break down or dissociate from the particles during snRNP purification. In the present report, the method used minimizes the chances of polypeptide degradation and the results obtained would not be affected by the disruption of fragile particles. The cells were lyophilized before they were broken, and the samples were not exposed to water until they were heated in SDS immediately before electrophoresis. Alternatively, the additional polypeptides might be tightly associated with the snRNP population that pellets with chromatin and nucleoli after nuclei are broken by sonication and centrifuged during other protocols, and not with the snRNPs that remain in the supernatant. snRNPs are purified from this supernatant (3). The discarded chromatin/nucleolar pellet contains about half of the cellular snRNA content (3), which is mainly, if not exclusively, in snRNP form (12). Then, the starting material for snRNP purification contains about half of the cellular snRNP content, while there is no evidence that the protein composition of the snRNP populations in the chromatin/nucleolar pellet is identical to that in the supernatant. The presence of cytoplasmic proteins which react with anti-Sm or anti-(U1)RNP antibodies has not

been described before in adult somatic cells. In addition, their apparent molecular weights differ from those of similarly reactive nuclear proteins. The possibility cannot be ruled out that some antibodies possessing non-snRNP or cross-reactive specificities might be present in the monoclonal anti-Sm and polyclonal anti-(U1)RNP antibody preparations used in the present work. However, it seems unlikely that this would be the explanation for all the additional nuclear and cytoplasmic polypeptides we have detected.

After our nonaqueous cell frationation procedure, virtually none of the molecules of mature U1, U2, U4, U5 and U6 RNA were found in the cytoplasmic fraction (11). This was true also in the current cell fractionations (Fig. 2, lane 8; and data not shown). Therefore, proteins detected in immunoblots of these cytoplasmic fractions probed with anti-Sm antibodies may be located in the cytoplasm *in vivo*. It seems reasonable to assume that the 28K protein detected with monoclonal anti-Sm antibodies in the cytoplasmic fraction is the same polypeptide as that found in the nucleus. Then, it is estimated by densitometry of the protein autoradiographs and RNA fluorographs that the amount seen in the cytoplasmic fraction (Fig. 1, lane 4) represents ~7% of the cell content. We did not determine if nuclear contamination of the cytoplasmic fraction (usually <3%) could totally account for this percentage. We do not know why monoclonal anti-Sm antibodies secreted by the same hybridoma cell line reacted well with a 16K protein in protein gel blots from partially purified snRNPs (2), but this was not the case with blots from a total nuclear fraction (Fig. 1, lane 9). It seems unlikely that it was caused by poor transfer of small proteins under our conditions, since lactoglobulin A (18.4K) and cytochrome c (12.3K) were electroblotted as efficiently as larger proteins (Fig. 2, lanes 5 and 6).

During *Xenopus laevis* development, fully grown oocytes and pre-gastrulation embryos accumulate snRNP proteins in the cytoplasm (23). In contrast, the present data show that somatic human cells do not have large cytoplasmic pools of the polypeptides that bear Sm or RNP antigenic determinants and are present in U snRNPs. These experiments tell us nothing about the snRNP pro-

teins that do not react directly with anti-Sm or anti-RNP antibodies. Our results suggest that adult somatic human cells may contain more populations of polypeptides carrying Sm or RNP antigenic sites than has been anticipated, both in the nucleus and cytoplasm, including some proteins that might not be full-time constituents of snRNPs.

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