Characterization of Heterogeneous Nuclear RNA-Protein Complexes In Vivo with Monoclonal Antibodies

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Exposure of cells to UV light of sufficient intensity brings about cross-linking of RNA to proteins which are in direct contact with it in vivo. The major [35 S]methionine-labeled proteins which become cross-linked to polyadenylated heterogeneous nuclear RNA in HeLa cells have molecular weights of 120,000 (120K), 68K, 53K, 43K, 41K, 38K, and 36K. Purified complexes of polyadenylated RNA with proteins obtained by UV cross-linking in intact cells were used to immunize mice and generate monoclonal antibodies to several of these proteins. Some properties of three of the proteins, 41K, 43K, and 120K, were characterized with these antibodies. The 41K and 43K polypeptides are highly related. They were recognized by the same antibody (2B12) and have identical isoelectric points ($pI = 6.0 \pm 0.2$) but different partial peptide maps. The 41K and 43K polypeptides were part of the 40S heterogeneous nuclear ribonucleoprotein particle and appear to correspond to the previously described C proteins (Beyer et al., *Cell* 11:127-138, 1977). A different monoclonal antibody (3G6) defined a new major heterogeneous ribonucleoprotein of 120K. The 41K, 43K, and 120K polypeptides were associated in vivo with both polyadenylated and non-polyadenylated nuclear RNA, and all three proteins were phosphorylated. The monoclonal antibodies recognized similar proteins in human and monkey cells but not in several other vertebrates. Immunofluorescence microscopy demonstrated that these proteins are segregated to the nucleus, where they are part of a fine particulate nonnuclear structure. In cells extracted in situ with nonionic detergent, all of the 41K and 43K polypeptides were associated with the nucleus at salt concentrations up to 0.5 M NaCl, whereas the 120K polypeptide was completely extracted at this NaCl concentration. A substantial fraction of the 41K and 43K polypeptides (up to 40%) was retained with a nuclear matrix—a structure which is resistant to digestion with DNase I and to extraction by 2 M NaCl, but the 41K and 43K polypeptides were quantitatively removed at 0.5 M NaCl after digestion with RNase.

The proteins which are associated with heterogeneous nuclear RNA (hnRNA) to form ribonucleoprotein complexes (hnRNPs) are thought to be involved in the packaging and processing of hnRNA (for a review, see reference 35). Current models of hnRNP structure based primarily on morphological (5, 6, 8, 15, 28, 40, 41) and sedimentation (7, 22, 33, 34, 44, 46, 55) studies of material released from nuclei by RNase digestion (22, 33, 34, 46, 55) or by mild sonication (7, 22, 44) suggest a "beads on a string" structure, with 40S particles forming the beads. There is some evidence that the 40S particles are associated with hnRNA in a sequence-discriminating manner (8, 48). The major components of the 40S particles are proteins with molecular weights in the 30,000 to 40,000 range (7, 22, 33, 34, 46, 55), classified as A, B, and C proteins (7, 30). The possibility that the 40S monomer fraction is actually heterogeneous and contains numerous additional proteins has also been raised (17, 49). Other proteins which can be isolated in vitro with hnRNA and are not part of the 40S particles have also been described, including those which are preferentially associated with the polyadenylate [poly(A)] segment (25, 36, 44, 47, 50). In the absence of other data, the criteria of cosedimentation or of other in vitro copurification techniques, such as oligodeoxythymidylate [oligo(dT)]-cellulose chromatography, must be considered only to be suggestive, because adventitious associations of proteins with RNA may occur (1, 18, 43). Furthermore, genuine RNP proteins may dissociate during such in vitro procedures. A different and more stringent definition of RNP proteins which overcomes these

shortcomings involves identification of proteins in direct contact with RNA in vivo by UV cross-linking in intact cells (13, 14, 38, 39, 51, 53, 54). This technique makes it possible to isolate the RNA of interest under protein denaturing conditions so as to ascertain that the proteins which do copurify with it are genuine RNP proteins, in that they must have been tightly associated with the RNA in vivo to become cross-linked to it by UV light. However, without additional probes for these proteins, the information about them and about their role in RNA metabolism remains limited. This report outlines an approach which combines the specificity of UV cross-linking in vivo for identification and isolation of genuine RNP proteins with the probing power of monoclonal antibodies. Purified UV-cross-linked complexes of poly(A)⁺ RNA with proteins were obtained from HeLa cells by UV irradiation of live cells and were injected into mice. The spleen lymphocytes from immunized animals were fused with myeloma cells to generate a library of hybridoma colonies secreting monoclonal antibodies to the RNA-associated proteins. The properties and intracellular localization of some of the major hnRNP proteins which were studied with these antibodies are described.

MATERIALS AND METHODS

Cell culture and labeling. HeLa cells were grown in monolayer culture in Dulbecco modified Eagle medium containing 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere. Cultures were supplemented with penicillin-streptomycin and used at subconfluent densities. Cells were labeled with [35 S]methionine at 10 μ Ci/ml for 4 h in methionine-free medium containing 2% undialyzed fetal calf serum.

UV irradiation. Irradiation of cell culture dishes was

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FIG. 1. Proteins cross-linked in vivo to poly(A)⁺ hnRNA and to poly(A)⁺ mRNA by UV irradiation of intact cells. HeLa cells were labeled with [³⁵S]methionine and exposed to UV light for 3 min. The poly(A)⁺ RNPs were prepared from the nuclear (hnRNP) and cytoplasmic (messenger RNA [mRNP]) fractions and digested with RNases, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

carried out in phosphate-buffered saline (PBS) containing Ca²⁺ and Mg²⁺ at room temperature as was recently described (13). The 15-W germicidal lamp was placed 4.5 cm away from the cell monolayer, and exposure was for 3 min.

Preparation of poly(A)⁺ RNPs. After UV irradiation, the PBS was removed and the cells were allowed to swell for 5 min in ice-cold RSB (10 mM Tris-hydrochloride, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂) containing 0.5% aprotinin (Sigma Chemical Co.), 1 µg of pepstatin A per ml, and 1 µg of leupeptin per ml. Triton X-100 was added to a final concentration of 0.5%, followed by 0.5% deoxycholate and 1% Tween 40, and the cells were homogenized by four passages through a 25-gauge needle. Nuclei and cytoplasmic fractions were separated by low-speed centrifugation. The cytoplasmic fraction was adjusted to 1 mM EDTA, 1% mercaptoethanol, and 0.5% sodium dodecyl sulfate (SDS). After heating at 90°C for 5 min, rapid chilling, and addition of LiCl to 0.5 M, the cytoplasmic extract was incubated for 15 min with oligo(dT)-cellulose (type 3; Collaborative Research) with constant agitation. The oligo(dT)-cellulose was then packed in a column, washed with >10 column volumes of binding

buffer (10 mM Tris-hydrochloride pH 7.4, 500 mM LiCl, 1 mM EDTA and 0.5% SDS). The performance of the column was monitored by liquid scintillation counting. The eluted poly(A)⁺ material (in 10 mM Tris-hydrochloride, pH 7.4, 1 mM EDTA and 0.05% SDS) was reheated to 65°C for 5 min, and the oligo(dT)-cellulose chromatography was repeated. When the poly(A)⁺ hnRNA fraction from UV-irradiated cells was prepared, procedures were similar, except that the nuclear fraction was used after DNase I digestion (50 µg/ml for 15 min at 37°C), the buffers contained 10 mM vanadyl-adenosine (VA) as RNase inhibitor (2, 10), and heating of samples before the first oligo(dT)-cellulose chromatography was only to 65°C. The DNase I (DPFF grade; Worthington Diagnostics) was treated with iodoacetamide (56) and further purified on an aminophenylphosphoryl-uridine-2'(3')-phosphate-agarose column to remove RNase contamination (37). The poly(A)⁺ material was precipitated overnight at -20°C with 3 volumes of ethanol.

RNase digestion of poly(A)⁺ RNP. The poly(A)⁺ material was pelleted by centrifugation at 12,500 × g and resuspended in 75 µl of 10 mM Tris-hydrochloride (pH 7.4) containing 1 mM CaCl₂, and digestion with RNase was carried out with 25 µg of pancreatic RNase A (Worthington) per ml and 400 U of micrococcal nuclease (P-L Biochemicals) per ml for 60 min at 37°C. To inhibit possible traces of protease, the pancreatic RNase was preboiled, and aprotinin (0.5%), pepstatin A (1 µg/ml), and leupeptin (1 µg/ml) (Sigma) were included in the digestion mixture. After the RNase digestion, the proteins were precipitated by addition of 3 volumes of ethanol at -20°C for at least 2 h.

Gel electrophoresis. Protein samples were electrophoresed on a SDS-containing discontinuous polyacrylamide gel electrophoresis system (SDS-PAGE) (13). The separating gel was prepared from a stock of 33.5% acrylamide and 0.3% *N,N'*-bisacrylamide to a final concentration of 12.5% acrylamide. The separating gel buffer contained 0.38 M Tris-hydrochloride (pH 9.1). The stacking gel was prepared from

FIG. 2. Mini-immunoblot analysis (3 by 5 cm) of the sera of mice immunized with UV-cross-linked HeLa poly(A)⁺ RNPs. The immunoblotting was carried out with total cell UV-cross-linked poly(A)⁺ RNPs as antigen. Sera dilutions were 1:250. Procedures were as described in the text.

a stock of 30% acrylamide and 0.44% *N,N'*-bisacrylamide to a final acrylamide concentration of 4% in 0.125 M Tris-hydrochloride (pH 6.8). Both gels contained 0.1% SDS and were polymerized with ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine. The electrode tank buffer was 25 mM Tris–192 mM glycine containing 0.1% SDS. Samples were prepared by boiling for 3 min in SDS sample buffer (0.125 M Tris-hydrochloride [pH 6.8], 2% SDS, 5% β -mercaptoethanol, 10% glycerol, and bromophenol blue). After electrophoresis of 35 S-labeled material, the gels were stained with Coomassie blue and impregnated with PPO (2,5-diphenyloxazole), and fluorography was performed by using preflashed X-ray films (29).

Preparation of monoclonal antibodies to UV-cross-linked RNPs. The poly(A)⁺ material from UV-irradiated HeLa cells (nuclei and cytoplasm) which eluted from the oligo(dT)-cellulose column after 2 cycles of chromatography was precipitated at –20°C with 3 volumes of ethanol. The cross-linked RNPs were digested with RNase A and with micrococcal nuclease as described above, except that incubation was only for 15 min, and reprecipitated. The pellet was dissolved in 0.5 ml PBS and mixed with an equal volume of complete Freund adjuvant, and 0.5 ml, corresponding to material from 50 UV-irradiated 10 cm tissue culture dishes, was injected intraperitoneally per BALB/c mouse. A similar booster injection in incomplete Freund adjuvant was given on day 14, and the mice were hyperimmunized by an intravenous injection of the same antigen in PBS on day 28. Mice were sacrificed 3 to 4 days later, the spleens were removed, and the lymphocytes were fused with Sp2/0 myelo-

FIG. 4. Species specificity of the antibodies against the hnRNP 41K, 43K, and 120K proteins. Cells grown in culture were washed twice with PBS and dissolved in SDS-sample buffer. A portion containing an equal number of cells was resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotting was carried out as described in the text. The nitrocellulose blot was first probed with ascites fluid of hybridoma 2B12 (A), and the same blot was subsequently reprobed with ascites fluid of hybridoma 3G6 (B). The cell lines used were: HeLa (human), CV-1 (monkey), 3T3 (mouse), CEF (chicken), and *Anolis carolinensis* lung fibroblasts (lizard).

FIG. 3. Immunoblot analysis of ascites fluids of hybridomas 2B12 and 3G6 against total HeLa cell material (T) and against UV-cross-linked poly(A)⁺ hnRNP (U).

ma cells. Hybridoma culture, cloning, and screening procedures were essentially as previously described (16, 26). Culture supernatants were initially screened for specific antibody-producing colonies by β -galactosidase-linked immunoassays and immunoblotting with the same material as the one injected as antigen. Positive colonies were expanded, recloned, and screened again with UV-cross-linked poly(A)⁺ material. Ascites fluids were prepared by intraperitoneal inoculation of hybridomas into pristane-primed BALB/c mice.

Immunoblotting and immunofluorescence. Blotting of proteins from polyacrylamide gels onto nitrocellulose paper was carried out by electrotransfer at 0.15 A in 50 mM Tris-glycine (pH 9.1) containing 20% methanol at room temperature for 6 to 12 h. The nitrocellulose blot was treated essentially according to Burnette (9), except that 0.5% gelatin was used instead of bovine serum albumin and the blot was incubated first with monoclonal antibody containing ascites fluid and then with 125 I-goat anti-mouse F(ab')₂. Dot blot analysis was carried out similarly, except that the samples were applied to the nitrocellulose directly with the aid of a dot blot apparatus (Schleicher and Schuell). Ascites fluid dilutions were 1:1000 for the 2B12 monoclonal antibody and 1:500 for the 3G6 monoclonal antibody. Immunofluorescence was carried out by using a Zeiss Photomicroscope III on cells cultured on glass cover slips and fixed with 2% formaldehyde for 30 min at room temperature, followed by 5 min in acetone at –20°C. Ascites fluid dilutions were as described above for immunoblotting. Detection of the mouse antibodies was with fluorescein isothiocyanate-conjugated goat-anti-mouse F(ab')₂ (Cappel Laboratories) used at a 1:50 dilution in 1% BSA in PBS.

Immunoprecipitation. Labeled cells were dissolved in PLB (1% Triton X-100–0.5% deoxycholate–0.1% SDS–0.5%

FIG. 5. Dot blot analysis of control un-cross-linked (untreated), UV-cross-linked (UV), and RNase-treated UV-cross-linked (UV + RNase) nuclear RNPs with 2B12 and 3G6 after sedimentation on SDS-sucrose gradients. Nuclei were prepared from UV-irradiated and from unirradiated HeLa cells. The nucleoplasmic fraction (postchromatin and postnucleolar fraction) was prepared from sonicated nuclei as previously described (44), SDS and β -mercaptoethanol were added to 0.5% and 1%, respectively, and the samples were heated to 65°C for 5 min. Samples (0.5 ml) were applied onto 15 to 30% sucrose gradients in 10 mM Tris-hydrochloride (pH 7.4)–10 mM EDTA–100 mM NaCl containing 0.2% SDS. Sedimentation was for 10 h at 23°C at 25,000 rpm in a Beckman SW41 rotor. Twenty-three fractions (0.5 ml each) were collected from the bottom. Samples (30 μ l) of each fraction were spotted onto the nitrocellulose paper. RNase digestion of the UV + RNase sample was with 0.5 μ g of RNase A per ml for 15 min on ice.

aprotinin–1 mM EDTA in PBS) and incubated with 2.5 μ l of ascites fluid for 5 h at 4°C. Five microliters of second antibody, rabbit anti-mouse immunoglobulin G F(ab')₂ specific, was added with 70 μ l of Staph A (10% suspension; IgG-sorb from The Enzyme Center, Inc.) to trap the immunocomplexes (24), and incubation was continued for 1 h more on a rocker at 4°C. Immunocomplexes with Staph A were pelleted and washed 3 times with PLB. The proteins were released by boiling for 5 min in 25 μ l of SDS sample buffer and were analyzed by SDS-PAGE.

Detergent and salt extraction of cells in situ. HeLa cells cultured on 35-mm dishes were permeabilized for 15 min on ice with 500 μ l of PIMPS buffer (10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.8, 300 mM sucrose,

FIG. 6. Dot blot analysis of nuclear poly(A)⁺ RNP and poly(A)[−] RNP with the monoclonal antibodies 2B12 and 3G6. Rapidly sedimenting UV-cross-linked material from a SDS-sucrose gradient was pooled (Fig. 5, UV, fraction 6 through 16) and applied to an oligo(dT)-cellulose column (0.5 ml). Samples (200 μ l) of the flow-through [5 ml, poly(A)[−] fraction, pA[−]] and of the fraction that was eluted at low ionic strength [5 ml, poly(A)⁺ fraction, pA⁺] were spotted onto nitrocellulose paper and probed with 2B12 and 3G6.

100 mM KCl, 2.5 mM MgCl₂) (10) containing 1% Triton X-100, 0.5% aprotinin, 1 μ g of pepstatin per ml, and 1 μ g of leupeptin per ml. The soluble fraction was removed, and the monolayer was extracted with an additional 500 μ l of PIMPS containing various concentrations of NaCl (0.1 to 2.0 M) for 3 min on ice. DNase I (100 μ g/ml), RNase A (100 μ g/ml),

FIG. 7. Immunoblot analysis of the relationship of the 41K and 43K proteins to the C proteins of the 40S hnRNP particle. 40S hnRNP particles, a gift from Wallace LeStourgeon, were prepared from HeLa cell hnRNPs by RNase digestion and sedimentation as described by Beyer et al. (7). (A) Total HeLa cell material; (B) 40S hnRNP particles. The position of the C proteins in the 40 S hnRNP particle sample was confirmed by staining with Coomassie blue.

VA (10 mM), aurintricarboxylic acid (2.5 mM), or sodium tetrathionate (2 mM) was added to PIMPS during the permeabilization step. These two extraction fractions were combined and designated as the extracted fraction (E). Residual material was scraped into 1 ml of PIMPS and designated the R fraction. Samples (100 μ l) of the E and R fractions were precipitated at -20°C for several hours with 3 volumes of ethanol and analyzed by SDS-PAGE and immunoblotting.

RESULTS

The proteins which became cross-linked *in vivo* to poly(A)⁺ mRNAs and poly(A)⁺ hnRNAs upon UV irradiation of intact HeLa cells are shown in Fig. 1. The two sets are different; although some of the proteins may be common to both messenger RNPs and hnRNPs, most of the major ones are associated with one of the two. The 72,000-molecular-weight (72K) polypeptide which is cross-linked specifically to the poly(A) segment (G. Dreyfuss, Y. D. Choi, and S. A. Adam, submitted for publication) and the 50K polypeptide, as well as a few others, appear to be exclusive to cytoplasmic messenger RNPs. On the other hand, several proteins, such as the 41K, 43K, and 120K polypeptides, seem to be specific to hnRNPs. These findings are consistent with previous *in vitro* data (19, 27, 31, 47) and suggest that transcripts exchange the proteins with which they are associated in the nucleus upon transport to the cytoplasm. Some cross-linking of hnRNA to proteins which may correspond in molecular weight to the B proteins and possibly also to the A proteins (36 to 38K) described by Beyer et al. (7) as components of the 40S hnRNP particle was also seen. These cross-linking patterns are similar to those recently reported (13, 14, 39, 52, 54). The possibility exists that some of the bands are products of protein-protein cross-linking, although this is quite unlikely and is shown below not to be the case for several specific proteins. The

FIG. 8. Immunoblot of a two-dimensional gel electropherogram of HeLa cell nuclear material probed with ascites fluid of hybridoma 2B12. Isoelectrofocusing in the first dimension was according to O'Farrell (42) with a pH 3 to 10 ampholine gradient. The second dimension was a SDS-PAGE as described in the text.

FIG. 9. Partial peptide map of the [³⁵S]methionine-labeled 41K and 43K proteins. Cells were labeled with [³⁵S]methionine, and the 41K and 43K proteins were immunoprecipitated with monoclonal antibody 2B12 and electrophoresed by SDS-PAGE as described in the text. After autoradiography, the bands corresponding to 41K and 43K were excised from the dried gel and hydrated, and peptide mapping was carried out by using 0.1 μ g of V8 protease per sample as described by Cleveland et al. (12).

cross-linking is strictly dependent on UV light, and no protein was detected on the autoradiograms if UV light was not used. Under the UV irradiation conditions employed in this work, over 85% of the poly(A)⁺ hnRNA and the poly(A)⁺ mRNA were recovered from cells exposed to UV light (G. Dreyfuss, Y. D. Choi, and S. A. Adam, submitted for publication; S. A. Adam, and G. Dreyfuss, manuscript in preparation). To obtain additional information about these proteins and about RNP complexes, it was desirable to raise antibodies to at least some of the cross-linked proteins. Since a conventional scheme which involves purification of the individual proteins seemed enormously laborious, a different experimental strategy was adopted. The UV-cross-linked poly(A)⁺ material (both nuclear and cytoplasmic) selected from a large number of irradiated monolayers of HeLa cells were used for "shotgun" immunizations of mice. A test minigel immunoblot of the sera of five immunized mice against the UV-cross-linked material which was used for the injections indicated that the mice responded to the complex UV-cross-linked RNP immunogens (Fig. 2). A large number (1,800) of hybridoma colonies were obtained after fusions of splenic lymphocytes of three mice with the Sp2/0 myeloma line. The culture fluids of these colonies were screened against UV-cross-linked poly(A)⁺ material by β -galactosidase-linked immunoassay and immunoblotting and by immunofluorescence microscopy on HeLa cells. Approximately 210 colonies were judged positive by two or more assays. The positive colonies were transferred to 1-cm culture dishes, and when subconfluent they were either

FIG. 10. Immunoprecipitation of the 41K and 43K proteins (2B12) and the 120K protein (3G6) from HeLa cells labeled with $^{32}\text{P}_i$. Cells were labeled with $^{32}\text{P}_i$ at 50 $\mu\text{Ci}/\text{ml}$ in phosphate-free minimal essential medium containing 2% undialyzed fetal calf serum for 2 h. Immunoprecipitation was carried out as described in the text. Phosphatase inhibitors (10 mM NaF and 20 μM ZnCl_2) were included throughout the procedure. Immunoprecipitation with ascites fluid of a mouse inoculated intraperitoneally with the parent myeloma line Sp2/0 was included as a control.

frozen down or recloned for further study. Of the colonies which were selected and recloned, the monoclonal antibodies secreted by two, designated 2B12 and 3G6, which recognized three of the major proteins which are in contact with hnRNA in vivo (hnRNPs) are described here in more detail.

The hybridoma 2B12 recognized a 41K and a 43K protein, and the 3G6 hybridoma recognized a 120K protein (Fig. 3). Over 60 of the initial hybridoma colonies were positive for both the 41K and 43K proteins. Not one has been found so far which reacted only with one of the two, in spite of the fact that not all of the hybridomas recognized the same epitope. The two polypeptides therefore share common antigenic determinants and are likely to be related. Upon cross-linking and after RNase digestion, the proteins were converted to a form which migrated slightly slower in SDS-PAGE. This is consistent with an increase of 500 to 1,000 daltons, which may correspond to one or more RNase-resistant covalently bound nucleotides. When a greater amount of material was applied to the gel, and more so with increasing irradiation time, higher-molecular-weight forms of ca. 85K and 125K were detected by 2B12. Since corresponding bands are not detected in material from unirradiated cells, these forms are likely to be oligomers of 41K and 43K which result from UV cross-linking. The cross-linked

120K polypeptide comigrated with the native 120K polypeptide without an apparent increase in molecular weight, possibly because the gel electrophoresis system does not detect such a small increment as would result from one or several linked nucleotides in the high-molecular-weight region. Because of the similarity in the mobilities in SDS-PAGE of the uncross-linked and the cross-linked proteins, the 41K, 43K, and 120K polypeptides are not likely to be cross-linked to other proteins. The species distribution of the 41K, 43K, and 120K polypeptides was examined by immunoblotting (Fig. 4). The monoclonal antibodies recognized these proteins in primates but not in several other vertebrate species.

The association of the 41K, 43K, and 120K polypeptides with nuclear RNA can also be demonstrated by sedimentation experiments in SDS-containing sucrose gradients (Fig. 5). The nuclear fraction from HeLa cells was boiled in SDS and β -mercaptoethanol and sedimented on SDS-sucrose gradients to dissociate noncovalently bound proteins from RNA. The gradients were fractionated and the amount of the 41K, 43K, and 120K polypeptides was determined in each of the fractions by a protein dot blot assay. In the absence of pre-exposure to UV light, the proteins remained at the top of the gradients (untreated). However, preexposure of cells to UV light converted a substantial fraction of these proteins into heavier material which cosedimented with hnRNA (UV). In this more rapidly sedimenting material, the proteins were associated with RNA because this material was sensitive to RNase, which converted it to lighter sedimenting material (UV + RNase). The sedimentation profile of rapidly labeled [^3H]uridine nuclear RNA was the same with or without UV cross-linking (untreated and UV), and the profile of the proteins after UV cross-linking coincided with it, whereas after RNase digestion the RNA signal moved (as did the proteins) to the top of the gradient (data not shown). These findings provide additional and independent evidence, without oligo(dT)-cellulose chromatography, to demonstrate that the 41K, 43K, and 120K polypeptides are RNP proteins, since they are in contact in vivo with high-molecular-weight nuclear RNA.

The rapidly sedimenting RNA-containing UV-cross-linked material from the SDS-sucrose gradients was pooled (Fig. 5; UV, fractions 6 through 16) and applied to an oligo(dT)-cellulose column to analyze for poly(A) $^+$ and poly(A) $^-$ RNA. The flowthrough (polyA $^-$) and the fraction that was eluted at low ionic strength (polyA $^+$) were probed with the 2B12 and 3G6 monoclonal antibodies. The data (Fig. 6) demonstrate that the 41K and 43K proteins, as well as the 120K protein, are associated in vivo with both poly(A) $^+$ and poly(A) $^-$ RNA. Immunofluorescence data shown below (see Fig. 11) demonstrate that these proteins are not found in nucleoli and, therefore, the high-molecular-weight poly(A) $^-$ RNA with which they are associated is likely to be poly(A) $^-$ hnRNA rather than ribosomal RNA.

From the data described above, it seemed possible that the 41K and 43K polypeptides are similar to the C group proteins previously described by Beyer et al. (7), as the two proteins of the 40S hnRNP particles which remain associated with the hnRNA in vitro under salt conditions where the other particle proteins dissociate. This was examined by immunoblotting comparing total HeLa cell material with 40S hnRNP particles prepared as described by Beyer et al. (7) and kindly provided by Wallace LeSturgeon. Figure 7 shows that the monoclonal antibody 2B12 recognized the C proteins, which are the same as the 41K and 43K proteins, and therefore establishes that the C proteins of the 40S

FIG. 11. Immunofluorescence microscopy of HeLa cells with monoclonal antibodies 2B12 and 3G6. Procedures were as described in the text, and photomicrography was carried out with the Zeiss 63 Planapo objective. The left and right panels are Nomarski and fluorescent micrographs, respectively, of the same fields of cells. (A and B), 2B12 (anti-41K and 43K); (C and D), 3G6 (anti-120K).

hnRNP particle are in direct contact with hnRNA (both polyA⁺ and polyA⁻) in vivo.

The relative abundance of the 41K and 43K proteins appeared from both immunoblotting and [³⁵S]methionine labeling and immunoprecipitation to be constant. Densitometric scanning of immunoblots indicated that the ratio of 41K to 43K protein is ca. 2:1. The same ratio was maintained in every type of cellular and biochemical fractionation so far attempted from both human HeLa cells and monkey CV-1 cells. Densitometric scanning also showed that under the irradiation conditions used here about one-third (32%) of the 41K protein which is recognized by 2B12 became cross-linked to RNA (data not shown).

The availability of the antibodies made it possible to examine some of the properties of the 41K, 43K, and 120K proteins. Immunoblotting of two-dimensional gel electropherograms of proteins from HeLa cells probed with the 2B12 monoclonal antibody showed that the 41K and 43K polypeptides are acidic and have identical apparent pIs of 6.0 ± 0.2 (Fig. 8). Repeated attempts to determine the isoelectric point of the 120K polypeptide have not been successful because it does not penetrate the isoelectric focusing gel. The immunological data and the identical isoelectric points of the 41K and 43K polypeptides, in spite of their different apparent molecular weights in SDS-PAGE, suggests that

they might be closely related. This was explored further by partial peptide mapping. The [³⁵S]methionine-labeled, immunoprecipitated 41K and 43K polypeptides were excised from SDS-polyacrylamide gels and digested in situ with V8 protease, and the partial peptide maps were compared (12), (Fig. 9). The two proteins generated a staggered set of similar partial peptides and are therefore likely to be homologous. Additional information about the N- or C-terminal regions and of post-translational modifications will be necessary to further characterize the relationship of the two proteins. Labeling of cells with ³²P_i and immunoprecipitation of the proteins with the specific antibodies indicated that the 41K, 43K, and 120K polypeptides are phosphorylated in vivo (Fig. 10). Holcomb and Friedman have also recently found that the C proteins of hnRNPs are phosphorylated by casein kinase II-type enzyme (E. R. Holcomb and D. L. Friedman, personal communication).

The subcellular organization of the hnRNP 41K, 43K, and 120K proteins was examined with the monoclonal antibodies by immunofluorescence microscopy and immunoblotting of different subcellular fractions. Indirect immunofluorescence microscopy with the 2B12 and 3G6 monoclonal antibodies localized the proteins to the nucleus (Fig. 11). No fluorescence signal was found in the nucleolus. The nuclear stain appeared to be composed of numerous dense and fine

FIG. 12. Immunoblot analysis of the association of the 41K, 43K, and 120K proteins with nuclear structures. (A) Effect of different salt concentrations. HeLa cells were permeabilized in situ as described in the text and further extracted for 3 min with 0.5 ml PIMPS buffer containing the various concentrations of NaCl. Samples (100 μ l) of the extractable (E) and the residual (R) fractions were analyzed by SDS-PAGE and immunoblotting. Ascites fluids of 2B12 (1:1000) and 3G6 (1:500) were combined to probe the blots. (B) Effect of various treatments on the association of the 41K, 43K, and 120K proteins with nuclear structures at 2 M NaCl. HeLa cells were permeabilized in situ for 15 min in an ice-water bath with 0.5 ml of PIMPS buffer containing DNase I (100 μ g/ml), RNase A (100 μ g/ml), VA (10 mM), aurintricarboxylic acid (ATA) (2.5 mM), or sodium tetrathionate (NaTT) (2 mM) and were further extracted for 3 min with 0.5 ml of PIMPS containing 2 M NaCl. Other experimental details were as described in (A).

particles which were more obvious at higher magnifications. The association of the proteins with nuclear structures was examined by immunoblotting. HeLa cells in monolayer were extracted with 0.5% Triton X-100 (at 100 mM KCl), the soluble fraction was removed, and the detergent-insoluble residue of the cell monolayer was subjected to the various extraction conditions described in Fig. 12 and 13. The mode of association with nuclear structures of the 41K and 43K proteins and of the 120K protein is different. Much of the 120K protein was released at 100 mM NaCl (Fig. 12A), and the protein was susceptible to proteolysis, which generated a slightly smaller polypeptide which showed as a doublet on the immunoblots. Significant amounts (ca. 40%) of the 41K and 43K proteins were still associated with remnant nuclear structures even after DNase I digestion and extraction with 2 M NaCl (Fig. 12A). This DNase- and high-salt-resistant structure is operationally defined as the nuclear matrix (2, 3, 23). The amount of the 41K and 43K proteins which was retained at high salt concentration was somewhat variable and depended on the state of transcription in the cell (unpublished results). However, the 41K and 43K proteins were completely released at 0.5 M NaCl if RNase was included in the extraction buffer (Fig. 13). van Eekelen and van Venrooij (52) have identified two proteins of 41.5K and 43K which became cross-linked to hnRNA upon irradiation of nuclei or cells with UV light. They have suggested that these proteins correspond to the C proteins of hnRNPs and that these are associated with the nuclear matrix even after treatment with RNase (52). These findings are different from those de-

scribed here in that we found that the association of what are likely to be the same proteins with nuclear structures was sensitive to RNase (Fig. 13). To further examine the possible role of RNase-sensitive linkages in the association of 41K and 43K proteins with nuclear structures, extractions were also carried out in the presence of the RNase inhibitors VA (4) and aurintricarboxylic acid (20). Aurintricarboxylic acid did not have a marked effect on the amount of the 41K, 43K, and 120K proteins which was retained with the nucleus at high salt concentration. In contrast, in the presence of VA there was a complete retention in the nucleus of all three hnRNP proteins. The effect of VA may not necessarily be a consequence of its RNase-inhibitory activity. The disulfide cross-linker sodium tetrathionate, which was found to stabilize nuclear matrix structures (23), had only a slight effect on the amount of 41K, 43K, and 120K proteins which remained with the matrix. The various treatments described in Fig. 12 and 13 were monitored also by immunofluorescence microscopy, which confirmed that the retained proteins were confined to the nucleus. However, after DNase I digestion and salt extraction, the immunofluorescence signal was lower than that found by immunoblotting and was estimated to be only about 10% of the total (data not shown). Altogether, the immunoblotting data can only be considered semi-quantitative and would probably bias in favor of the nuclear matrix-retained fraction because the total amount of protein loaded per (R) lane was lower than that in the soluble (E) fraction, and consequently binding to the nitrocellulose paper could have been higher.

FIG. 13. HeLa cells were permeabilized and digested in situ with 100 μ g of RNase A per ml for 15 min on ice in 0.5 ml of PIMPS buffer as described in the legend to Fig. 12 and then were extracted for 3 min with 0.5 ml of PIMPS buffer containing the various NaCl concentrations. E, extracted fraction; R, residual fraction.

DISCUSSION

We described here the preparation of monoclonal antibodies to genuine hnRNP proteins—those which are in direct contact with hnRNA in vivo. The RNP proteins were identified by UV cross-linking in intact cells, and the cross-linked complexes were further used to immunize mice and obtain hybridoma colonies secreting monoclonal antibodies against the specific proteins. This approach is general and may be useful for any RNA-associated proteins in vivo. One obvious limitation of this approach is that it may fail to include all of the RNP proteins; there may, theoretically, be additional proteins which are actually associated with the RNA in the cell which could, for a variety of chemical and steric reasons, not become cross-linked by UV light. This is possible particularly for proteins (if such exist) which interact with the RNA only through the phosphates or through the sugars rather than through the bases which are the moieties which become photoactivated. It is also theoretically possible that some of the proteins for which antibodies may be obtained are not cross-linked to the hnRNA directly but to another RNA (e.g., small RNA), which in turn is cross-linked by UV light to the hnRNA either directly or via another protein. This latter possibility cannot be completely ruled out, but it is not likely to be the case for the proteins described here because no small RNA was detected after protease digestion of UV cross-linked poly(A)⁺ hnRNP complexes (unpublished results).

Previous reports have described studies of hnRNPs with antibodies (11, 21, 32, 45). Jones and colleagues (21) have

prepared antibodies to the “core” proteins (30,000 to 40,000 daltons) of the 40S hnRNP particles from mouse cells in chickens and have carried out extensive morphological studies with them. Christensen et al. (11) have prepared antibodies to the 40S hnRNP particles and have demonstrated the presence of these proteins in *Drosophila* polytene chromosomes. Risau et al. (45) have recently reported on the packaging and nonpackaging hnRNP proteins in *Drosophila* using monoclonal antibodies. All of these studies have used in vitro isolated hnRNPs as sources of antigen.

Of the monoclonal antibodies which were obtained to poly(A)⁺ hnRNA, the properties of two, designated 2B12 and 3G6, and of the proteins they recognize are described. 2B12 recognizes two related 41K and 43K proteins, which are shown to be identical to the C proteins of 40S hnRNP particles (7, 30). The data obtained with 2B12 therefore establishes that the C proteins are associated with both poly(A)⁺ hnRNA and poly(A)⁻ hnRNA in vivo, that they are segregated to the nucleus, and that the two C proteins are related to each other. The antibody data also indicate that the C proteins are phosphorylated and that they are a distinct group from the other groups of proteins (A and B) which make up the 40S particle. Furthermore, whereas the A and B proteins are basic (7), and C proteins are acidic (Fig. 8), and several antibodies which react with the 41K and 43K proteins at different epitopes do not react with the A and B proteins. The relative amounts of the 41K and 43K proteins are constant in many different cellular and biochemical fractions, and they therefore appear to behave like two subunit polypeptides of one multisubunit protein or of a larger structure.

The hnRNP 120K protein has not been previously described, although proteins of high molecular weight associated with hnRNA in vitro (7, 22, 25, 31, 36, 47, 49, 50) and in vivo (13, 14, 39, 52) have been identified. It is shown to be a genuine hnRNP protein because it is cross-linked to high-molecular-weight nonnucleolar nuclear RNA in vivo. The portion of hnRNA with which it is associated and its relationship to the A, B, and C proteins of the 40S particles are not yet known. Sedimentation analysis in sucrose gradients of hnRNPs prepared by sonication from HeLa cell nuclei without UV cross-linking also shows that the 120K protein cosediments with hnRNPs (Y. D. Choi, H. R. Choi, A. Reicin, and G. Dreyfuss, manuscript in preparation). The 120K protein is a nuclear phosphoprotein which is associated with both poly(A)⁺ hnRNA and poly(A)⁻ hnRNA.

One of the interesting points to emerge from the studies described here relates to the association of the 41K and 43K proteins with nuclear structures. A substantial fraction of the 41K and 43K proteins (ca. 40% by immunoblotting) is retained in situ with a nuclear matrix structure which is resistant to digestion with DNase I and to extraction by 2 M NaCl. These proteins are, however, completely released at moderate salt concentration after RNase digestion. This finding is different from that reported recently by van Eekelen and van Venrooij (52), in which two proteins which appear to be the same as the 41K and 43K proteins described here were described as being associated with the nuclear matrix even after RNase digestion and therefore were proposed to be the proteins which anchor hnRNA to the nuclear matrix. The apparent discrepancy may, in part, be the result of differences in the preparation of the nuclear matrix. Whereas the nuclear matrix fraction is prepared here in situ, van Eekelen and van Venrooij (52) used isolated nuclei and carried out the digestion with RNase after extraction with 2 M NaCl. Kaufman et al. (23) have shown that after exposure

to high salt concentration nuclei are no longer sensitive to RNases. It is also possible that van Eekelen and van Venrooij (52) did not detect the release of the C proteins from the matrix by RNase because they monitored the release by sedimentation of the nuclear matrix out of a buffer of very low ionic strength (10 mM) rather than, as is the case here, at 100 mM NaCl or higher. It may also be possible that the identification by these authors of the C proteins without specific antibodies is not accurate or that our antibodies may recognize only a subpopulation of the C proteins. It is interesting that the RNase inhibitor VA increases dramatically the amount of the 41K and 43K proteins and also of the 120K protein which is retained with salt-washed nuclear structures. This, however, is not necessarily the result of its RNase inhibitory activity.

The specific antibodies to genuine RNP proteins should help in the study of their role in the synthesis, processing, and function of RNA with *in vitro* systems and microinjection techniques. Furthermore, the approach described here for the production of specific antibodies to proteins which are in direct contact with polynucleotides *in vivo* may be of general usefulness to the study of nucleic acid-binding proteins.

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