hnRNA and Its Attachment to a Nuclear Protein Matrix

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ABSTRACT   In this study, DNA-depleted nuclear protein matrices are isolated from HeLa S3 cells. These nuclear matrices consist of peripheral laminae, residual nucleoli, and internal fibrillar structures. High molecular weight, heterogeneous nuclear RNA (hnRNA) is quantitatively associated with these structures and can be released intact only by affecting the integrity of the matrices. It is, therefore, concluded that hnRNA is part of a highly organized nuclear structure.

By irradiation of intact cells or isolated nuclear matrices with ultraviolet light, proteins tightly associated with hnRNA can be induced to cross-link with the RNA. Performing the cross-linking in vivo is an extra guarantee that only hnRNA-protein (hnRNP) complexes existing in the intact cell are covalently linked. Such hnRNP complexes were isolated and purified under conditions that completely dissociate nonspecific RNA-protein complexes.

By comparison of the hnRNP found in nuclear matrices and the published data on the composition of hnRNP particles, it was found that the so-called hnRNP "packaging" proteins (32,000–38,000 mol wt) were not efficiently cross-linked to hnRNA by UV irradiation. They were, however, present in the matrix preparations, bound to hnRNA, because they were released from nuclear matrices after ribonuclease treatment of these structures. On the other hand, two major hnRNPs (41,500 and 43,000 mol wt) were efficiently cross-linked to hnRNA. These proteins were not released by ribonuclease treatment, which suggests that they are involved in the binding of hnRNA to the nuclear matrix.

Although in the last decade much knowledge has been acquired about hnRNA at the molecular level, little is known about the role of various cellular structures and components in transcription, RNA processing, and RNA transport to the cytoplasm. To investigate such a role, studies have been undertaken to identify protein and RNA molecules that interact with heterogeneous nuclear RNA (hnRNA). hnRNA can be extracted from nuclei in the form of hnRNA-protein (hnRNP) particles (reviewed by Heinrich et al. [1] and Van Venrooij and Janssen [2]) that have a repeating subunit structure composed of 200–300 Å spherical particles connected by a ribonuclease-sensitive strand (3, 4). Small nuclear RNA seem to be integral parts of hnRNP particles (5–8). The protein composition of hnRNP particles is very complex and seems to be related to the isolation procedure used. Although nonspecific binding of proteins to the RNA during the isolation of the particles has never rigorously been excluded, most workers (9–11) agree on the presence of some predominant proteins in the 30,000–44,000 mol wt range.

The isolation of hnRNP particles mostly involves nuclear breakage (for example, by sonication) or extraction of the nuclei with buffer solutions for prolonged periods at a relatively high temperature. In general, the amount of hnRNP released from the nuclei seems to depend very much on the degree of nuclear disintegration achieved (2).

Detergent-treated nuclei from eukaryotic cells, depleted of their DNA, retain a residual insoluble protein structure referred to as nuclear skeleton or nuclear matrix (12–20). This nuclear matrix consists of: (a) a peripheral layer of a complex of nuclear pores and connecting lamina, (b) residual nucleolar structures, (c) internal fibrillar protein structures. The peripheral pore-lamina complexes have been purified (21–23) and shown to contain three major polypeptides in the 60,000–70,000 mol wt range (21). These proteins are located at the periphery of nuclei in interphase cells (as shown by immunofluorescence) and not in the internal nuclear structures (24, 25). Very little is known about the protein composition of the internal nuclear structure. It is evident, however, that it is much more complex than the composition of the pore-lamina complexes (14–16, 26). Some of the matrix proteins could be involved in the binding of hnRNA, because two groups of investigators have reported that in a variety of cells the hnRNA is quantitatively associated with the nuclear matrix (13, 17, 18, 26). Herman and co-workers (17) showed with electron microscope radioautography

that in HeLa cell nuclei the hnRNA was associated with the internal protein structure. These authors suggested that hnRNA is essential for the integrity of the nuclear matrix. This conclusion, however, was not supported by the results of Pogo and collaborators who found that in liver cells, Krebs ascites cells, and erythroleukemic cells the hnRNA is associated with the nuclear matrix (18, 26) but that ribonuclease treatment removes the hnRNA without disintegration of the internal structure (26). Furthermore, Herlan and co-workers (19) showed that also pre-rRNA and rRNA are almost quantitatively attached to the nuclear skeletons of *Tetrahymena* macronuclei. Because Long et al. (26) noticed that hnRNA can be released from the internal protein matrix only by disrupting these structures, it is very well possible that hnRNP particles, as discussed above, are products of nuclear disintegration caused by mechanical forces or enzymatic action.

In this study, the isolation and characterization of nuclear matrices of HeLa S3 cells are described. In an attempt to establish which proteins are involved in the association of hnRNA with the nuclear matrix, isolated matrices and intact cells were subjected to UV irradiation to cross-link hnRNA to some of its associated proteins. Evidence is presented that the same set of proteins was cross-linked to hnRNA irrespective of whether the cross-linking was performed on intact cells or on isolated matrices. Our results suggest that the major cross-linked proteins are involved in the binding of hnRNA to the nuclear matrix.

MATERIALS AND METHODS

Tissue culture media and sera were purchased from Flow Laboratories Ltd., Irvine, Scotland. All chemicals used were of analytical grade. Buffers were boiled in the presence of 0.02% diethylpyrocarbonate and then autoclaved. Radiochemicals were obtained from the Radiochemical Centre, Amersham, England.

Cell Growth and Labeling

HeLa S3 cells were grown in suspension at 37°C at densities ranging between 0.5 and 1 × 10⁶ cells/ml on Eagle's Minimal Essential Medium supplemented with 5% newborn calf serum, 5% fetal calf serum, and vitamins. Mycoplasma tests were run regularly and were always negative. Cell doubling occurred every 24 h. For preferential labeling of hnRNA, cells were concentrated 10-fold (5–10 × 10⁶ cells/ml) and incubated with 5 μCi/ml [5,6-³H]uridine (40 Ci/mmol) for 10 min. Cellular protein was labeled by incubating the cells overnight with 2 μCi/ml [³⁵S]methionine (±1,000 Ci/mmol) at densities of 1–2 × 10⁶ cells/ml. For the first 2 h, the cells were incubated in medium without unlabeled methionine, then 0.1 vol of complete medium was added. Another volume of complete medium was added 1 h before harvesting the cells.

Cell Fractionation and Purification of Nuclear Matrices

Cells were harvested on frozen NKM (130 mM NaCl, 5 mM KCl, 7.5 mM MgCl₂), pelleted by centrifugation (5 min at 800 g), washed once with NKM solution, and pelleted again. The cell pellet was suspended in cold reticulocyte suspension buffer (RSB) (0.01 M NaCl, 0.01 M Tris [pH 7.4], 1.5 mM MgCl₂, 0.5 mM PMSC [phenylmethylsulfonyl chloride]) and after addition of 0.5% Na-deoxycholate (DOC) and 1% Tween 40 (Atlas Powder Co., Wilmington, Del.) homogenized by 20 strokes of a motor-driven Teflon pestle in a Potter-Elvehjem tissue homogenizer (Kontes Co., Vineland, N. J.). The nuclei were pelleted (5 min at 800 g) and washed once with RSB. The nuclear pellet was resuspended in HRSB (0.11 M NaCl, 0.01 M Tris pH 7.4, 0.01 M MgCl₂, 0.5 mM PMSC) at a density of 1 × 10⁸ nuclei/ml and incubated with 500 μg/ml DNase 1 for 15 min at 20°C. 0.5–1.0 ml of the digest was transferred to a tube filled with 2 ml of 2.6 M sucrose (underlayer) and 10 ml of 1 M sucrose in HRSB and centrifuged for 15 min at 1,000 g at 4°C. The DNA-depleted nuclei, concentrated on the heavy sucrose layer, were gently resuspended in 5 ml of 0.4 M (NH₄)₂SO₄, 30 mM Tris (pH 7.4), 10 mM MgCl₂, spun down (5 min at 800 g), and resuspended in RSB. Suspensions of the high-salt-extracted nuclear matrices obtained in this way were

used for UV-induced cross-linking experiments and for hnRNA and hnRNP complex isolation.

Before use, DNase 1 (Worthington Biochemical Corp., Freehold, N. J.; DPFF quality, "RNase free") was freed from contaminating traces of RNase activity by affinity chromatography over 5'-UTP-agarose (Sigma Chemical Co., St. Louis, Mo.) as described by Smith et al. (27). DNase 1 was tested for ribonuclease activity by incubating ³H-labeled 28S rRNA for 1 h at 37°C with 50 μg/ml DNase 1. The purified DNase 1 used in all our experiments did not degrade 28S, rRNA when tested in this way.

UV-light-induced Cross-linking

UV-induced RNA-protein (RNP) cross-linking was performed on nuclear matrices suspended in RSB or on intact cells suspended in NKM buffer at concentrations of 10⁸ matrices or cells/ml, following the procedure of Wagenmakers et al. (28). Samples to be irradiated were transferred into small quartz tubes, which were put into a bigger quartz tube filled with ice water. During irradiation the cells or matrices were gently shaken every 3 min. Irradiation was performed in a wooden box covered on the inside with aluminum foil. On each of the four edges of the box, one 15-W germicidal tube (Philips TUV) was fixed. The distance between sample and lamps was ~4 cm. 70% of the irradiation energy was emitted at 253.7 nm. The radiation dose at this wavelength received by the sample was determined by ferrioxalate actinometry (29) and found to be 8,000 J/m² (10²² quanta/m²) per minute.

The amount of RNP cross-linking was determined by SDS-phenol/chloroform extraction (30, 31). This extraction procedure separates RNA from RNP complexes because free RNA remains in the aqueous phase, while covalent RNP complexes and free protein move into the interphase. Irradiated nuclear matrices or nuclear matrices from irradiated cells containing ³H-labeled hnRNA were boiled in 1% SDS for 2 min and centrifuged for 5 min at 5,000 g, and a phenol/chloroform extraction was performed on the clear supernate. The organic phase plus the interphase were re-extracted twice with a buffer containing 0.5% SDS, 50 mM Tris (pH 7.4), 100 mM NaCl, and 5 mM EDTA. Aliquots from the combined aqueous fractions and the organic phase plus interphase were mixed with Picofluor-15 (Packard Instrument Co., Downers Grove, Ill.) and counted. When, after repeated washings with SDS-containing buffer, the organic phase and interphase were diluted with ethanol and the pellets obtained after centrifugation were treated with proteinase K (100 μg/ml, 1 h, 37°C) and re-extracted with phenol/chloroform, then hnRNA was quantitatively present in the water phase. This indicates that the presence of RNA radioactivity in the combined organic and interphase indeed was attributable to complexing with protein. Neither irradiation up to 10 min nor subsequent proteinase K treatment caused significant degradation of the hnRNA, as judged from RNA sedimentation profiles in glycerol gradients.

Isolation of Covalent RNP Complexes

A discontinuous sucrose-D₂O-H₂O gradient was used for the rapid and easy isolation of covalent RNP complexes. The gradient consisted of a 1-ml underlayer of 80% sucrose in D₂O (density 1.35 g/cm³) and on top of this a layer of 3.5 ml of 1.25 M sucrose in a D₂O/H₂O mixture (density 1.20 g/cm³) containing 10 mM Tris (pH 7.4) and 0.1% SDS. 0.5-ml samples containing 1% SDS were layered on these gradients and centrifuged for 18 h at room temperature at 240,000 g in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The middle layer has a density that prevents free protein from sedimenting into the gradient, while the underlayer prevents the RNP complexes (densities between 1.2 and 1.35 g/cm³) from pelleting. In this way, free protein could be separated from protein covalently linked to RNA. To avoid contamination of the lower fractions with the bulk of the radioactivity present in the top fractions, the gradients were fractionated starting from the bottom. In a control experiment (not shown), it was found that when nuclear extracts from irradiated or unirradiated cells were analyzed on this type of gradient, >70% of the [³H]uridine-labeled hnRNA could be recovered from the high-density region of the gradient after an 18-h run as described above.

Analysis of Proteins

Samples for SDS polyacrylamide gel electrophoresis were prepared as follows. In the case of proteins covalently bound to RNA, the RNA moiety was digested by a 1-h incubation at 37°C with 400 U/ml micrococcal nuclease (P-L Biochemicals, Inc., Milwaukee, Wis.) and 25 μg/ml RNase A (Sigma Chemical Co.) in 10 mM Tris (pH 7.4) containing 1 mM Ca²⁺. This procedure degrades >99.9% of [³H]RNA bound to protein, irrespective of the ³H-nucleoside precursor used. Free protein was precipitated with 7% TCA. The precipitations were pelleted, washed twice with acetone, and dissolved in sample buffer (2% SDS, 10% glycerol,

and 5% β-mercaptoethanol). SDS polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (32).

Electron Microscopy

Suspensions of nuclei or nuclear matrices were fixed overnight in 0.5% glutaraldehyde, 0.1 M Na-cacodylate (pH 7.4), washed overnight at 4°C with 0.1 M Na-cacodylate buffer, and then postfixed in 2% osmium tetroxide in 0.1 M Palade buffer for 1 h. Samples were stained for 2 h in 0.5% uranyl acetate in 0.1 M Palade buffer, dehydrated with ethanol followed by propylene oxide, and finally embedded in Epon. Sections were further stained for 30 min with 3.3% uranyl acetate and for an additional 15 min with 1.3% lead citrate. Examination was performed in a Philips EM 301.

RESULTS

Isolation and Characterization of HeLa Nuclear Matrices

Mechanical homogenization of HeLa cells in a DOC/Tween mixture followed by a wash with RSB (see Materials and Methods) results in a nuclear preparation that is essentially free of cytoplasmic contamination as judged by electron microscopy. The double membrane of the nuclei is solubilized by the detergent treatment, and most of the soluble nuclear molecules are removed. Nonsolubilized remnants of the membrane structures and the pore-lamina complex together form a dark-staining peripheral nuclear layer. Inside the nuclei the presence of nucleoli is obvious, whereas the chromatin seems to be rather randomly distributed as a result of the low-salt treatment. From these nuclei, nuclear matrices as shown in Fig. 1A could be prepared, following a procedure in which the DNA is digested and gently extracted (see Materials and Methods).

Although almost all DNA and associated proteins were removed by the digestion and subsequent extraction procedures (Fig. 2), an extensive internal nuclear structure can still be seen in the matrices. This internal structure consists of residual nucleoli and fibrillar structures. It was striking that, although >99% of the DNA was removed from the matrices, generally ~80% of the hnRNA cosediments with intact matrices (Fig. 2). These results are in agreement with those of Herman et al. (17) and Miller et al. (18). It should be noted here that most of the RNA released from the nuclei during both extraction steps (Fig. 2) could be pelleted from the nuclear extracts by low-speed centrifugation, which suggests that it was bound to very large structures, most probably fragments of nuclear matrices disrupted during the extraction. When the matrices subsequently were treated with RNase A, most though not all of the hnRNA could be removed from these structures. Electron microscope observation then showed that the RNase-treated matrices had a morphological appearance very similar to that of the nontreated matrices (Fig. 1B). This is not surprising, as RNP complexes proved to be only a minor component of nuclear matrices (see below). Consequently, it must be concluded that in HeLa cells hnRNA seems not to be essential for the integrity of the nuclear matrix structures. Our results, therefore, corroborate those of Miller et al. (18), who concluded, also on the basis of electron microscope observation of RNase-treated matrices, that in liver cells the internal nuclear structure does not depend on RNA for its integrity.

Characterization of the hnRNA Associated with the Nuclear Matrices

Rapidly labeled hnRNA isolated from the nuclear matrices and analyzed on glycerol gradients as described in the legend

of Fig. 3 was found to be degraded when commercially available "RNase-free" DNase 1 was used. Purification of the DNase over a 5'-UTP-agarose column (27), however, removed the contaminating ribonuclease in a simple one-step procedure. From matrices prepared with the purified DNase 1, high molecular weight, rapidly labeled hnRNA could be prepared. The sedimentation profile of matrix-bound hnRNA, after denaturation, was compared with that of hnRNA isolated from whole nuclei (Fig. 3). Because the sedimentation profiles are almost identical, it can be concluded that nuclear matrices, prepared as described in Materials and Methods, contain most
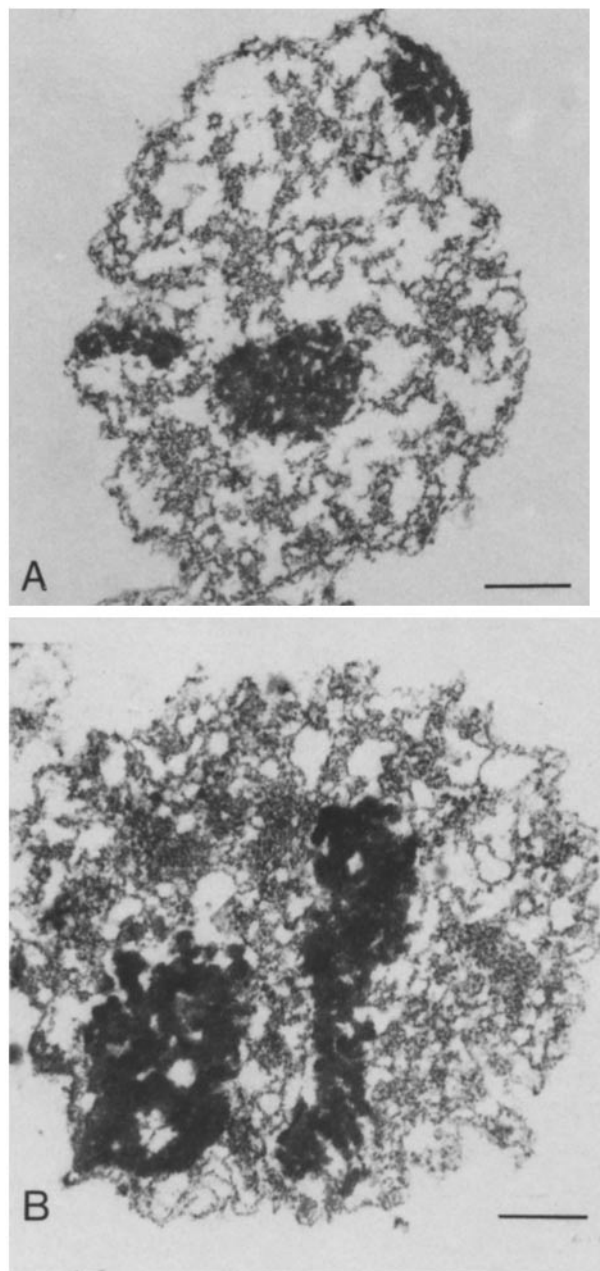

FIGURE 1 (A) Electron micrograph of a HeLa cell nuclear matrix. Nuclear matrices were prepared from HeLa cell nuclei after complete removal of the chromatin by DNase I digestion and subsequent extraction of the digested material (see Materials and Methods). (B) As in A but after isolation nuclear matrices were treated with RNase A (100 μg/ml, 20 min, 25°C). Bars, 1 μm. A, × 11,000; B, × 11,500.

of the high molecular weight hnRNA chains in an apparently undegraded form.

UV Cross-linking of hnRNA to Associated Proteins

To study the interaction of hnRNA with nuclear matrix proteins, UV irradiation was used to induce cross-linking between RNA and protein (30, 31, 33). To be sure that the in vivo situation was being studied, we irradiated not only isolated matrices but also intact cells.

First, the UV dose necessary for adequate cross-linking was determined. For this purpose cells and nuclear matrices, labeled with [³H]uridine in their hnRNA, were irradiated for various periods of time, and the amount of cross-linked hnRNA was determined by SDS-phenol/chloroform extraction (see Materials and Methods). After 5 min of irradiation of cells (Fig. 4A), as well as of isolated nuclear matrices (Fig. 4B), most of the hnRNA had already been cross-linked to protein. Irradiation of the isolated matrices for >5 min resulted in considerable losses of cross-linked RNA that became insoluble even in 1% SDS at 100°C (Fig. 4B). On the other hand, when intact cells were irradiated for longer periods of time no significant portion of the hnRNA was present in the insoluble material (Fig. 4A). However, as it is known that prolonged irradiation tends to induce protein-protein cross-linking (34; and our own observations), in our further experiments an irradiation time of only 3 min was used. This is equivalent to a radiation energy at 253.7 nm of 2.4×10^4 J/m² (28) and is sufficient to cross-link 50–80% of the hnRNA to proteins.

Isolation of Cross-linked hnRNP Complexes

Two types of density gradients were used to separate hnRNA and free protein from cross-linked hnRNP complexes. In our first experiments, CsCl density gradients were used. Nonirradiated and irradiated matrices containing ³⁵S-labeled protein and ³H-labeled hnRNA were treated with 1% Sarkosyl at 100°C, and the soluble fraction was centrifuged to equilibrium

in CsCl-Sarkosyl gradients. Most of the hnRNA in the nonirradiated sample equilibrated at a buoyant density >1.6 g/cm³. The buoyant density of the hnRNA from irradiated matrices was shifted to 1.38–1.48 g/cm³. The most likely explanation for this finding is that protein was cross-linked to the hnRNA, because ³⁵S-protein label of irradiated matrices also showed a peak in the same density region (data not shown).

In more recent experiments, a sucrose-D_2O-H_2O gradient was used to isolate cross-linked hnRNP complexes. The gradient conditions employed in these experiments were such that >70% of the (cross-linked) hnRNA was found in the high-density region of the gradient when extracts of irradiated or nonirradiated cells were analyzed (see Materials and Methods). The method thus allows a reasonable yield of cross-linked hnRNP complexes in a simple and convenient manner. Cells labeled with [³⁵S]methionine were irradiated for 3 min (in vivo) before preparation of the nuclear matrices. These were extracted with 1% SDS at 100°C, and the soluble fraction containing ~90% of the hnRNA was loaded onto a sucrose-D_2O-H_2O density gradient (Fig. 5B). The same procedure was applied to unirradiated cells (Fig. 5A) and irradiated (in vitro) isolated nuclear matrices (Fig. 5C). The results show that when no UV irradiation was employed, an insignificant fraction of the total matrix protein (<0.1%) migrated into the high-density region of the gradient (Fig. 5A). Only in the samples from irradiated material did labeled protein appear in that part of the gradient with densities >1.20 g/cm³ (fractions 1–8). Generally, 1.0–1.5% (irradiated cells, Fig. 5B) or 2.5–4.0% (irradiated matrices, Fig. 5C) of the total nuclear matrix protein was found in the high-density region of the gradient. It is most likely that we are dealing here with covalent RNP complexes, because these complexes are ribonuclease sensitive. When matrices from irradiated cells were treated with RNase A before the extraction with SDS, also <0.1% of the total matrix protein label migrated into the high-density region of the gradient (Fig.

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                              ⎧ [³H]RNA: fast label (10 min)
            HeLa nuclei ⎨ [¹⁴C]DNA: steady-state label
                              ⎩
              ↙        ↓        ↘
    Control        DNase 1      DNase 1, RNase A
   incubation      digestion        digestion
       +               +                +
   extraction      extraction       extraction
       ↓               ↓                ↓
 RNA    87%          80%              16%
 DNA    97%          <1%              <1%
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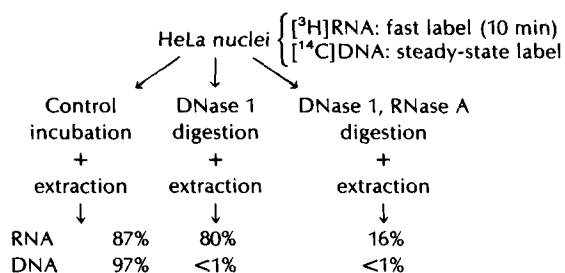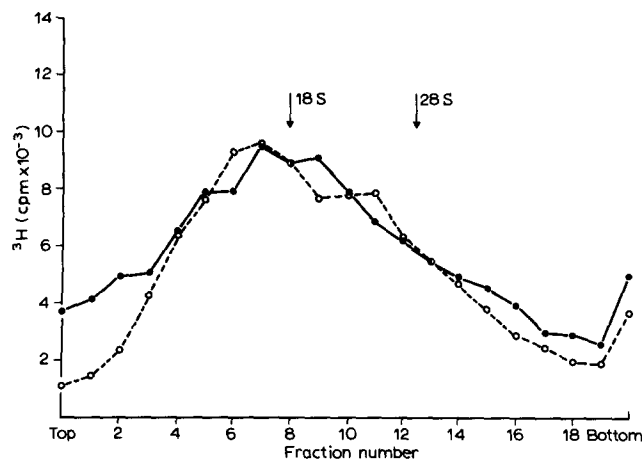
FIGURE 2 Analysis of HeLa nuclear structures after various enzymatic treatments. HeLa cell nuclei, prepared as described in Materials and Methods, were incubated with DNase 1 (500 µg/ml, 15 min, 20°C) and RNase A (10 µg/ml, 15 min, 20°C) as indicated. The digested material was removed by a two-step extraction procedure. The first extraction was carried out by layering the digest on 1 M sucrose in HRSB buffer and subsequent centrifugation for 15 min at 800 g. The pellet of extracted nuclei was then resuspended in 0.4 M (NH₄)₂SO₄ buffer and pelleted again. DNA was labeled by incubating the cells overnight at a density of 10⁶ cells/ml in the presence of 0.5 µCi/ml [¹⁴C]thymidine (57 mCi/mmol). Then the hnRNA was labeled by concentrating the cells 10-fold and incubating them in the presence of 5 µCi/ml [5,6-³H]uridine for 10 min at 37°C. The figures given represent percentages of the total acid-precipitable radioactivity initially present in the nuclei.

FIGURE 3 Glycerol gradient analysis of hnRNA isolated from HeLa nuclei and HeLa nuclear matrices (skeletons). [³H]hnRNA was isolated from purified nuclei or from nuclear matrices by hot phenol extraction at 55°C, as described by Long et al. (26), and subsequently precipitated by the addition of ethanol. The RNA pellets were dissolved in a small volume of water, denatured at 63°C in 25% dimethyl sulfoxide, 50% dimethylformamide, 10 mM EDTA, and 0.2% SDS for 2 min as described by Dubroff and Nemer (45), and applied to 10–41% isokinetic glycerol gradients containing 10 mM Tris, pH 7.4, and 0.1% SDS. Centrifugation was for 3 h in a Beckman SW50.1 rotor (Beckman Instrument Co.) at 300,000 g at 20°C. ●, Nuclei; ○, nuclear matrices.

5 *D*). In subsequent experiments, the high-density RNP complexes were further purified by oligothymidylic acid [oligo-(dT)]cellulose chromatography in the presence of SDS.

Oligo(dT)-Cellulose Chromatography of Covalent hnRNP Complexes

Because we were interested to know which proteins were cross-linked to hnRNA, [³⁵S]methionine-labeled covalent hnRNP complexes obtained via sucrose-D₂O-H₂O gradients were further fractionated on oligo(dT)-cellulose as described by Aviv and Leder (35) for mRNA. This procedure implies the presence of SDS and 0.5 M NaCl in the binding buffer before elution with a low-salt buffer. When total unfractionated SDS extracts from unirradiated nuclear matrices were applied to oligo(dT)-cellulose columns, no detectable ³⁵S-labeled proteins could be eluted with the low-salt buffer, indicating that chromatography in the presence of SDS minimizes nonspecific binding of proteins to the column material. On the other hand, when the pooled ³⁵S-labeled covalent hnRNP complexes from the sucrose-D₂O-H₂O gradients were applied (fractions *1-8* of Fig. 5 *B* and *C*), ~16-23% of the protein label was found and subsequently eluted from the columns. The binding of these labeled proteins was completely RNase sensitive. When re-chromatography (again in the presence of SDS) was done, >90% of the labeled proteins were re-bound to the oligo(dT)-cellulose and could be re-eluted with the low-salt buffer. These results clearly indicate that ~16-23% of the proteins were indeed covalently linked to poly(A)+hnRNA. It is interesting

to note here that we found that generally 17-25% of the rapidly labeled hnRNA associated with nuclear matrices was poly-adenylated. Consequently, it can be assumed that the non-bound fraction of the protein was cross-linked to poly(A)—hnRNA rather than to rRNA or pre-rRNA. This assumption could be substantiated by glycerol gradient analysis of the cross-linked RNA. From cells, labeled with [³H]uridine for 3 h and subsequently irradiated for 3 min, nuclear matrices were prepared. Cross-linked RNA was isolated from the phenol layer plus interphase fraction (see Materials and Methods), treated with proteinase K, denatured, and analyzed on glycerol gradients as described in the legend of Fig. 3. The results showed that the RNA components of both poly(A)+ and poly(A)—RNP complexes were heterogeneous in size and not of ribosomal nature. The possibility that the poly(A)—hnRNA complexes could be derived from poly(A)-containing complexes as a result of RNA-chain breakage during the boiling of the matrices in SDS cannot totally be excluded. However, when hnRNA was prepared from matrices by use of the standard procedure of phenol extraction at 55°C (thus omitting the boiling in SDS), a similar percentage of poly(A)-containing hnRNA (~20%) was found, indicating that boiling in SDS does not generate significant amounts of poly(A)—RNA.

Analysis of Matrix-associated hnRNP Complexes

The proteins associated with hnRNA in the nucleus have been identified only after isolation of hnRNP particles from disrupted nuclei under conditions that did not always exclude
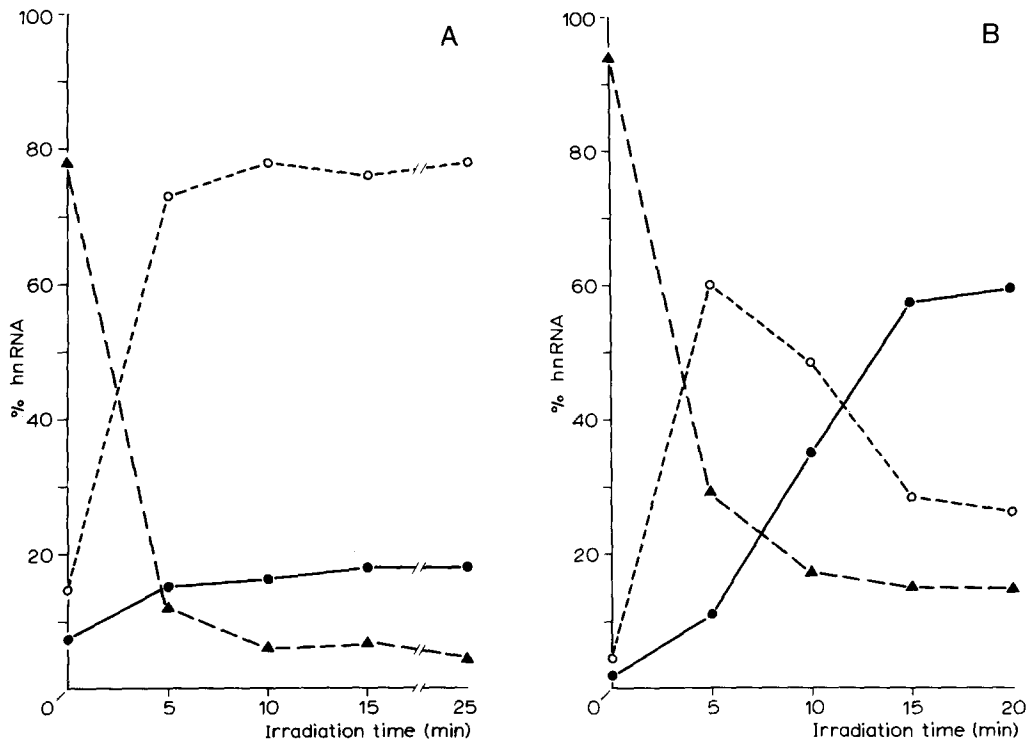

FIGURE 4 Quantitation of hnRNA cross-linking as a function of irradiation time. Labeling of HeLa cells and preparation of nuclear matrices was performed as described in Materials and Methods. In the type of experiment depicted in *A*, intact cells were irradiated for various periods of time. From these irradiated cells, nuclear matrices were prepared and analyzed. In a second type of experiment, nuclear matrices were isolated and subsequently irradiated (*B*). The nuclear matrices were boiled in 1% SDS for 2 min and centrifuged for 5 min at 5,000 *g*, and the radioactivity in the pellet, referred to as SDS-insoluble material, was measured. A phenol/chloroform extraction (see Materials and Methods) was performed on the supernate to fractionate the hnRNA into a cross-linked (organic phase plus interphase) and a non-cross-linked fraction (water phase). O, Percentage of [³H]hnRNA present in organic phase plus interphase; ●, percentage of [³H]hnRNA found in SDS-insoluble material; ▲, percentage of [³H]hnRNA present in aqueous phase.

the possibility of nonspecific binding of proteins to exposed regions of RNA during the isolation procedure. As a consequence, data on the number of proteins present in hnRNP particles vary considerably (1, 2), although the presence of a group of low molecular weight polypeptides (32,000–44,000 mol wt) as the main proteins of hnRNP particles has been generally accepted now.

It is of interest to know which of these hnRNPs are involved in the binding of hnRNA to the nuclear matrix. Considering the fact that ribonuclease digestion does not disrupt the nuclear matrix structure (see above), it can be expected that the protein(s) associated with hnRNA but not involved in the binding of hnRNA to the matrix will be released during ribonuclease
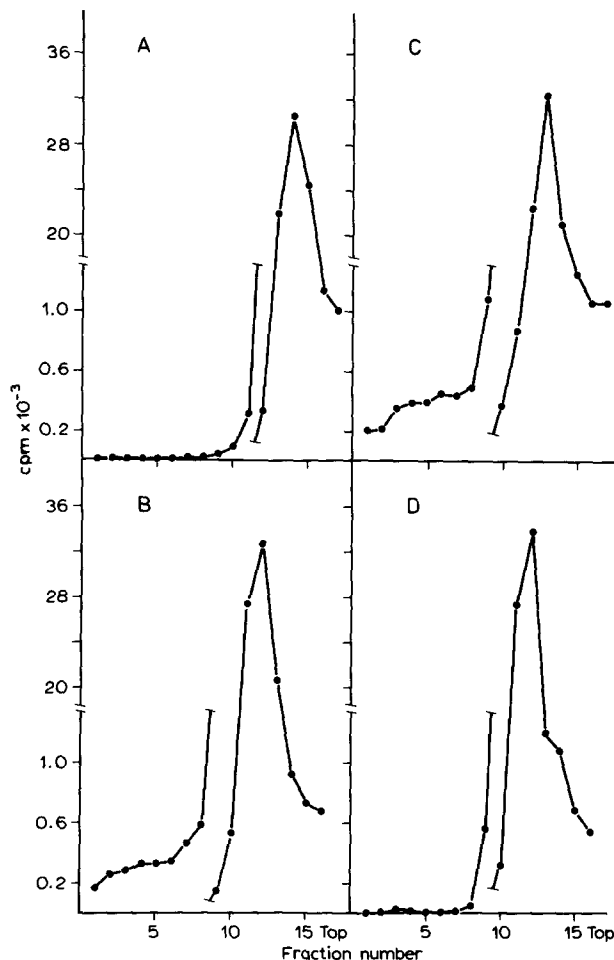
treatment. In such experiments, matrices were prepared from cells labeled with [^{35}S]methionine and incubated with and without RNase A at 37°C (see legend of Fig. 6). Although >80% of the hnRNA was released from the matrices by the ribonuclease treatment (cf. Fig. 2), only 1–2% of the total ^{35}S-labeled proteins were released from the nuclear matrices, indicating that hnRNA-associated proteins are only a very minor fraction of the total matrix protein. Therefore, the protein patterns of matrices before and after incubation look very similar (Fig. 6, lanes 2 and 3). Nevertheless, some prominent polypeptides were released from the matrices as a result of the ribonuclease treatment. A number of them were found in the 33,000–38,000 mol wt range and probably are identical (at least in mobility on SDS gels) with some of the main hnRNP subparticle proteins (10).

In attempts to determine which matrix protein(s) are involved in the binding of hnRNA, we irradiated [^{35}S]methionine-labeled cells with UV light to cross-link hnRNA in vivo to proteins tightly associated with it. Nuclear matrices from

FIGURE 5   Analysis of unirradiated and irradiated nuclear matrices on discontinuous sucrose-D$_2$O-H$_2$O gradients. Cells were labeled overnight with [^{35}S]methionine as described in Materials and Methods. One portion of the cells was irradiated for 3 min, and then nuclear matrices were prepared. From a second portion of cells, nuclear matrices were prepared and then irradiated for 3 min. From the third (control) portion of cells, unirradiated nuclear matrices were prepared. The nuclear matrix preparations were extracted with 1% SDS for 2 min at 100°C, and the cleared extracts were layered onto sucrose-D$_2$O-H$_2$O gradients prepared as described in Materials and Methods. Centrifugation was carried out for 18 h at 240,000 g in a Beckman SW50.1 rotor at 20°C. (A) 1% SDS extract of nuclear matrices from control, unirradiated cells. (B) 1% SDS extract of nuclear matrices from cells irradiated for 3 min (in vivo cross-linking). (C) 1% SDS extract of nuclear matrices irradiated for 3 min after isolation of the matrices (in vitro cross-linking). (D) As for B, but after isolation of the nuclear matrices from the irradiated cells, these were incubated with RNase A (100 μg/ml) for 20 min at 37°C.
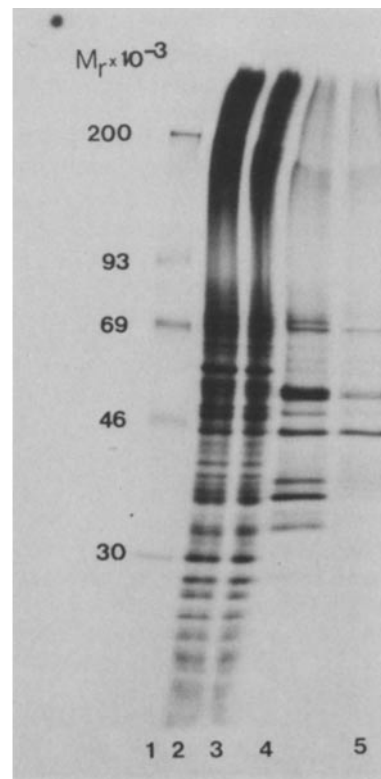
FIGURE 6   Analysis of proteins released from nuclear matrices by RNase A. HeLa cells were labeled with [^{35}S]methionine and nuclear matrices were prepared as described in Materials and Methods. One half of the matrix preparation was incubated with RNase A (100 μg/ml, 20 min, 37°C) in 1 ml RSB buffer; the other half was incubated in 1 ml of RSB buffer only. After the incubation, the nuclear matrix suspensions were centrifugated (5 min at 5,000 g). The pellets, containing the residual nuclear matrix proteins, and the supernates, containing the proteins released during the incubation, were prepared for SDS gel electrophoresis as described in Materials and Methods. The figure shows a fluorograph of a 10–18% gradient gel. Lane 1, molecular weight marker proteins; lane 2, nuclear matrix proteins from control-incubated matrices; lane 3, nuclear matrix proteins from RNase A-incubated matrices; lane 4, proteins released during the incubation of nuclear matrices with RNase A; lane 5, proteins released during the control incubation of HeLa nuclear matrices.

these cells were dissolved by boiling in 1% SDS and cross-linked poly(A)+ and poly(A)–hnRNP complexes were purified as described above. The cross-linked hnRNP complexes were extensively treated with a mixture of RNase A and micrococcal nuclease before analysis of the proteins on SDS polyacrylamide slab gels was carried out. Fig. 7 shows a fluorogram of such an analysis. No hnRNA-associated proteins could be isolated from nonirradiated cells or matrices (Fig. 7, lanes 2 and 3). From irradiated matrices or from matrices of irradiated cells, a typical pattern of hnRNA-associated proteins was obtained (Fig. 7, lanes 4–7), which proteins are a subset of the matrix proteins (cf. Fig. 6, lane 2, and Fig. 7, lane 8). It is evident that the patterns of proteins cross-linked to poly(A)+hnRNA (Fig. 7, lanes 4 and 5) and poly(A)–hnRNA (Fig. 7, lanes 6 and 7) are strikingly similar. In both cases, the major cross-linked polypeptide has a molecular weight of 42,000. In other experiments, in which a different type of acrylamide gel was used, this major cross-linked protein was

separated into two polypeptides of 41,500 and 43,000 mol wt, respectively (Fig. 8, lane 1).

These results were confirmed by another type of experiment. Covalent RNP complexes, labeled in the RNA moiety by incubating the cells for 15 min in medium supplemented with [^{3}H]adenosine, [^{3}H]uridine, and [^{3}H]cytidine (see legend to Fig. 7) before harvesting and UV irradiation, were isolated as described above for [^{35}S]methionine-labeled complexes. Poly(A)-containing hnRNP complexes were purified by oligo(dT)-cellulose chromatography and treated exhaustively with a mixture of RNase A and micrococcal nuclease. When the oligo(dT)-cellulose eluate from nonirradiated cells was digested, no TCA-precipitable radioactivity was obtained. After irradiation, however, 0.05% of the RNA label originally present could be precipitated by TCA by virtue of its covalent binding to protein. Knowing that 50–80% of the hnRNA was cross-linked to protein and assuming the average length of an RNA strand to be ~2,500 nucleotides, this means that one to
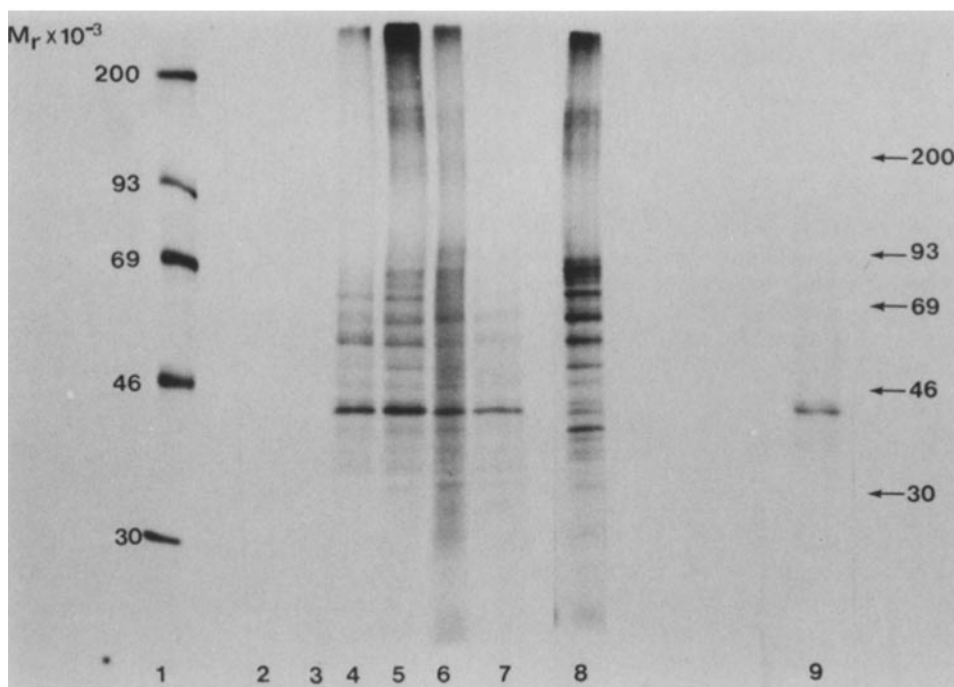

FIGURE 7   Analysis of proteins cross-linked to poly(A)+ and poly(A)–hnRNA by UV irradiation. HeLa cells were labeled overnight with [^{35}S]methionine as described in Materials and Methods. One part of the cells was irradiated for 3 min, and from these irradiated cells nuclear matrices were prepared. From a second portion of cells, nuclear matrices were prepared and then irradiated. From a third portion of cells, unirradiated matrices were isolated. The three batches of nuclear matrices were extracted with 1% SDS (2 min, 100°C), and the soluble fractions were applied to discontinuous sucrose-D₂O-H₂O gradients. To the fractions containing the cross-linked hnRNA-protein complexes (corresponding to the fractions 1–8 of Figs. 5A–C), NaCl was added to a final concentration of 0.5 M. Via oligo(dT)-cellulose chromatography (35), the cross-linked material was fractionated into poly(A)+ and poly(A)–hnRNP. The latter fraction was dialyzed overnight against 10 mM Tris, pH 7.4. The dialysed poly(A)–hnRNP fraction and the low-salt oligo(dT)-cellulose eluate, containing poly(A)+hnRNP, were treated with RNase A (25 µg/ml) and micrococcal nuclease (400 U/ml) in the presence of 1 mM Ca²⁺ for 1 h at 37°C. After the nuclease digestion, the proteins in the samples were precipitated with 7% TCA, washed twice with acetone, dissolved in sample buffer, and subjected to polyacrylamide gel electrophoresis. In a similar type of experiment, HeLa cells (2 × 10⁶ cells/ml) were labeled for 15 min with [^{3}H]adenosine, [^{3}H]uridine, and [^{3}H]cytidine (each 2 µCi/ml), then harvested and irradiated for 3 min. Poly(A)-containing hnRNA-protein complexes were isolated and digested with nucleases as described above for the [^{35}S]methionine-labeled complexes. The proteins in the samples were then prepared for SDS polyacrylamide gel analysis and fluorography. The figure shows a fluorograph of 10% polyacrylamide gels. Lane 1, molecular weight marker proteins (for lanes 2–8); lane 2, proteins covalently associated with poly(A)–hnRNA from unirradiated cells; lane 3, proteins covalently associated with poly(A)+hnRNA from unirradiated cells; lane 4, proteins cross-linked to poly(A)+hnRNA from cells irradiated with UV light for 3 min; lane 5, proteins cross-linked to poly(A)+hnRNA from matrices irradiated with UV light for 3 min; lane 6, as lane 5, but poly(A)–hnRNA; lane 7, as lane 4, but poly(A)–hnRNA; lane 8, total nuclear matrix protein; lane 9, proteins covalently associated with poly(A)+hnRNA from irradiated cells labeled by their residual [^{3}H]RNA components. Arrows indicate the positions of marker proteins, only for lane 9.
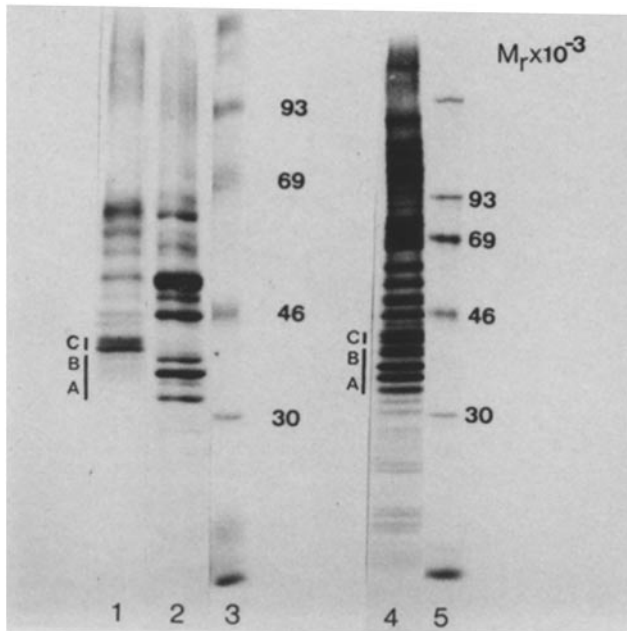
FIGURE 8 Comparison of hnRNP proteins with proteins released from nuclear matrices by RNase A digestion and proteins that can be cross-linked to poly(A)+hnRNA by UV irradiation of intact cells. The samples of [^{35}S]methionine-labeled proteins were prepared as described in the legends of Figs. 6 and 7. The figure shows a fluorograph of a 10–18% SDS polyacrylamide gel. Lane 1, proteins cross-linked to poly(A)+hnRNA isolated from cells irradiated with UV light for 3 min (compare Fig. 7, lane 4); lane 2, proteins released during the incubation of nuclear matrices with RNase A (compare Fig. 6, lane 4); lane 3, molecular weight marker proteins for the lanes 1 and 2; lane 4, crude preparation of [^{35}S]methionine-labeled proteins from HeLa cell 30S hnRNP particles, isolated following the method of Beyer et al. (10); lane 5, molecular weight marker proteins for lane 4. A, B, and C indicate the molecular weight ranges of the three groups of main hnRNP proteins that can be discerned in isolated hnRNP particles, according to Beyer et al. (10).

three nucleotides remained attached to proteins of the covalent poly(A)-containing hnRNP complexes. When these proteins were analyzed by gel electrophoresis, the fluorograph showed that the residual [^{3}H]radioactivity was associated with proteins of 41,500 and 43,000 mol wt (Fig. 7, lane 9). When, in the control experiment, the same procedure was applied to nonirradiated cells, no protein-associated ^{3}H label could be detected on the fluorograph (not shown). This result proves that these two proteins are directly associated with the hnRNA and not indirectly, for example, by disulfide bonds.

The 41,500 and 43,000 mol wt proteins were not released by the standard high-salt treatment during the preparation of nuclear matrices, as irradiation of isolated nuclear matrices cross-linked about the same amount of these proteins as did irradiation of whole cells. Both proteins were also not released during the RNase treatment of nonirradiated matrices (cf. Fig. 6). These facts and the finding that these proteins specifically can be cross-linked to hnRNA by UV irradiation of intact cells strongly suggest that the proteins are involved in the binding of hnRNA to the nuclear matrix.

DISCUSSION

The protein components constituting the internal part of the nuclear matrix have not been identified or localized yet, as has

been done for cytoskeletal proteins by, for example, immuno-fluorescence studies. However, several groups of workers using various kinds of isolation procedures have described the morphology of such structures in many types of cells (12, 16–18, 36). Besides that, evidence has been obtained that intranuclear matrix structures are involved in processes such as DNA replication (37), hnRNA attachment (17, 18), and specific hormone binding (38).

In this study we have presented a method to isolate nuclear matrices of HeLa S3 cells in which special care was taken to avoid damage by proteolytic or ribonuclease activities. The isolated nuclear matrices were composed of a peripheral layer and an internal matrix consisting of nucleolar and fibrillar structures. They were strikingly similar to nuclear matrices isolated from various types of cells by other workers (17, 18, 26). In our method of matrix isolation, the high-salt extraction to remove digested DNA (17) was preceded by a centrifugation of the DNase 1-treated nuclei over a sucrose layer because this evidently led to increased conservation of internal structures. It was confirmed that rapidly labeled hnRNA copurified with the matrices, and we were able to show that undegraded hnRNA could quantitatively be recovered from purified matrices (Fig. 3). The RNA did not seem to be essential for the integrity of the internal fibrillar structure (Fig. 1 B). The association of hnRNA with the nuclear matrices proved to be very stable, a finding that is in agreement with recent results of Long et al. (26). The hnRNA can be recovered from nuclear matrices only by disruption of these structures. Harsh treatments such as 8 M urea, 2 M KCl, 5% DOC, 10 M formamide, or prolonged sonication did not release the hnRNA from the matrix (26; and our own unpublished results). Because, for example, sonication or extraction with buffers is routinely used for the isolation of hnRNP particles from nuclei (1, 2), it should be realized that, among the proteins present in such preparations probably matrix proteins, involved in the binding of hnRNA, are also present.

To distinguish between the matrix proteins bound to hnRNA and the packaging proteins associated with the hnRNA but not with the matrix, we treated nuclear matrices with ribonuclease. Because the matrix itself is ribonuclease resistant, such a treatment releases only the proteins that are associated with the hnRNA and not with the matrix (generally 1–2% of the total matrix protein). Some prominent polypeptides were released specifically by the ribonuclease treatment (Fig. 6). Three to five of the released proteins were found in the 33,000–38,000 mol wt range and comigrated on SDS polyacrylamide gels with the group A and B proteins (32,000–34,000 and 36,000–37,000 mol wt, respectively) described by Beyer et al. (10) as being the main hnRNP subparticle proteins (see Fig. 8).

The matrix proteins involved in the binding of hnRNA were analyzed after their cross-linking to the RNA by UV irradiation. UV irradiation (at 254 nm) is known to induce covalent cross-linking of protein to DNA and to RNA, and the specificity of UV-induced cross-linking has been confirmed by many investigators (28, 39–43). It is generally agreed upon that a firm, noncovalent complex must be present at the time of irradiation to achieve covalent cross-linking (40–43). To corroborate the specificity of the association of hnRNA with the nuclear matrices, UV irradiation was performed on intact cells as well as on isolated nuclear matrices. Furthermore, the cross-linked complexes were purified in the presence of SDS to prevent copurification of noncovalently associated proteins. Although there also seems to be some cross-linking of predom-

inant proteins of the nuclear matrix preparations, two minor nuclear matrix components (41,500 and 43,000 mol wt) were very efficiently cross-linked to poly(A)+hnRNA as well as to poly(A)−hnRNA by both in vivo and in vitro irradiation (Figs. 7 and 8). In addition to that, the same type of experiment was also performed on hnRNP complexes labeled in the RNA moiety. After purification of the hnRNP complexes, the RNA was exhaustively digested away. The residual TCA-precipitable radioactivity was covalently attached to protein and sufficient in amount (about one to three nucleotides per protein molecule) to visualize these proteins after fluorography (Fig. 7, lane 9). The major proteins that were cross-linked to hnRNA in these experiments had apparent molecular weights of 41,500 and 43,000 (Fig. 8), and we found that these proteins comigrated on SDS polyacrylamide gels with the group C proteins (42,000–44,000 mol wt) described by Beyer et al. (10) as "interacting directly with hnRNA to form a smaller high salt-resistant complex." On the basis of salt dissociation studies on hnRNP particles, Beyer et al. (10) concluded that these group C proteins are more tightly associated with hnRNA than are the group A and B proteins. A protein with a similar molecular weight (40,000) was also described by Augenlicht et al. (44) in the human colon carcinoma cell line H2-29. They reported a nuclease-resistant complex of ~26 ribonucleotides and a major protein of 40,000 mol wt that was distinct from the major hnRNP protein in these cells, which had a molecular weight of 34,000.

The results depicted in Fig. 7 may suggest that other hnRNP proteins (for example, the 33,000–38,000 mol wt A and B proteins) were not cross-linked to the RNA during UV irradiation. However, recent experiments[1] have shown us that at least two additional polypeptides (corresponding in mobility on SDS gels to the B_1 and B_2 hnRNP core proteins) are cross-linked to hnRNP as well, although much less efficiently than are the C proteins. A possible explanation for this would be the fact that the A and B hnRNP core proteins are not as tightly associated with hnRNA as are the C proteins, as has been demonstrated by salt-dissociation studies (10). It is known that efficient cross-linking by UV irradiation occurs only when the interacting molecules are close and when their reactive groups are less than one bond length apart (39).

Our experiments, thus, indicate quite clearly that the 41,500 and 43,000 mol wt proteins are present in 0.4 M $(NH_4)_2SO_4$-washed nuclear matrices and are tightly associated with hnRNA. However, they are apparently not released by the ribonuclease treatment of these structures (Fig. 6). The most likely explanation for these results is to assume that hnRNA is bound to the nuclear matrix via these proteins.

As a conclusion we may state that our experiments indicate that there is a specific association of hnRNA with a nuclear protein matrix. The hnRNA in nuclear matrices is associated with a specific set of proteins that are very similar to the hnRNA-associated proteins found by other groups. Some of these hnRNA-associated proteins seem to function in the hnRNA–nuclear matrix binding. The hnRNA-containing nuclear matrix is, to a certain degree, an accurate representation of the in vivo situation, because hnRNA present in isolated matrices is associated with the same set of proteins as it is in the intact cell. Thus, the nuclear matrix may represent a very useful model structure for studies on the processing and transport of RNA in the nucleus.

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[1] C. A. G. van Eekelen. Manuscript in preparation.

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