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 a group 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 both 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 $1 \times 10^8$ cells/ml (13). Cells were harvested on frozen NKM (130 mM NaCl, 5 mM KCl, 7.5 mM MgCl$_2$, 75 mM Tris (pH 7.4) containing 1 mM Ca$^{2+}$) as described by Smith et al. (27). DNase 1 was tested for ribonuclease 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 H-labeled 28S RNA for 1 h at 37°C with 50 pg/ml DNase I. 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 $10^6$ 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 UV) 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/m$^2$ (10$^3$ quanta/m$^2$) 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 H-labeled hnRNA were boiled in 1% SDS for 10 min and centrifuged for 5 min at 5,000 g, and a phenol/chloroform extraction was performed on the clear supernatant. 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 pg/ml, 1 h, 37°C) and re-extracted with phenol/chloroform, then hnRNA was quantitatively present in the organic phase, which indicates that the presence of RNA radioactivity in the combined organic phase 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-D$_2$O-$H_2$O 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 D$_2$O (density 1.35 g/cm$^3$) and on top of this a layer of 3.5 ml of 1.25 M sucrose in a D$_2$O-$H_2$O mixture (density 1.20 g/cm$^3$) 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/cm$^3$) from sedimenting into the gradient. While the underlayer prevents the RNP complexes (densities between 1.2 and 1.35 g/cm$^3$) from sedimenting into the gradient.

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 pg/ml RNAse A (Sigma Chemical Co.) in 10 mM Tris (pH 7.4) containing 1 mM Ca$^{2+}$. This procedure degrades $>$99.9% of the RNA bound to protein, irrespective of the RNA-nucleoside precursor used. Free protein was precipitated with 7% TCA. The precipitations were centrifuged, washed twice with acetone, and dissolved in sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01 M Tris (pH 8.0), 10% glycerol, 0.1% SDS).

Cell Fractionation and Purification of Nuclear Matrices

Cells were harvested on frozen NKM (130 mM NaCl, 5 mM KCl, 7.5 mM MgCl$_2$, 75 mM Tris (pH 7.4) containing 1 mM Ca$^{2+}$) as described by Smith et al. (27). DNase 1 was tested for ribonuclease 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 H-labeled 28S RNA for 1 h at 37°C with 50 pg/ml DNase I. The purified DNase 1 used in all our experiments did not degrade 28S rRNA when tested in this way.
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. 1 A 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. 1 B). 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
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 $[^3]H$]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 $\times$ 10$^{-3}$ J/m$^2$ (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 $^{35}S$-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/cm$^3$. The buoyant density of the hnRNA from irradiated matrices was shifted to 1.38–1.48 g/cm$^3$. The most likely explanation for this finding is that protein was cross-linked to the hnRNA, because $^{35}S$-protein label of irradiated matrices also showed a peak in the same density region (data not shown).

In more recent experiments, a sucrose-D$_2$O-H$_2$O 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 $^{35}$S-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$_2$O-H$_2$O 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/cm$^3$ (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. 5C).
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, 1[^35]S-methionine-labeled covalent hnRNP complexes obtained via sucrose-D$_2$O-H$_2$O 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 3[^35]S-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 3[^35]S-labeled covalent hnRNP complexes from the sucrose-D$_2$O-H$_2$O 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 rechromatography (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 polyadenylated. 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
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 [35S]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 35S-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 [35S]methionine-labeled cells with UV light to cross-link hnRNA in vivo to proteins tightly associated with it. Nuclear matrices from

![Figure 5](https://example.com/figure5.png)  
**Figure 5.** Analysis of unirradiated and irradiated nuclear matrices on discontinuous sucrose-D2O-H2O gradients. Cells were labeled overnight with [35S]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-D2O-H2O 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 irradiated for 3 min after isolation of the matrices (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](https://example.com/figure6.png)  
**Figure 6.** Analysis of proteins released from nuclear matrices by RNase A. HeLa cells were labeled with [35S]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 supernatants, 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 [3H]adenosine, [3H]uridine, and [3H]cytidine (see legend to Fig. 7) before harvesting and UV irradiation, were isolated as described above for [35S]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](https://jcb.rupress.org)
cross-linked to poly(A)+hnRNA isolated from cells irradiated with radiation. No protein-associated 3H label could be detected in nuclear 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 stability of the internal fibrillar structure (Fig. 1B). The association of hnRNA with the nuclear matrices proved to be very strong, 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, 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 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-
nuclease-resistant complex of ~26 ribonucleotides and a major A protein with a similar molecular weight 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 HZ-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 have shown that at least two additional polypeptides (corresponding in mobility on SDS gels to the B1 and B2 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 not more 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 NH4SO4 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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3. Beyer et al. (10) concluded that these group C proteins were 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 HZ-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 have shown that at least two additional polypeptides (corresponding in mobility on SDS gels to the B1 and B2 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 not more 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 NH4SO4-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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