HETEROGENEOUS NUCLEAR RNA-PROTEIN FIBERS IN CHROMATIN-DEPLETED NUCLEI

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ABSTRACT

The heterogeneous nuclear RNA-protein (hnRNP) fibers in HeLa cell nuclei are visualized by a nuclear subfractionation technique which removes 96% of the chromatin in a single step and 99% in a two-step elution but leaves the bulk of the hnRNA complexed with the remnant nuclear structure or lamina. Both steady-state and newly synthesized (~15-s label) hnRNA are associated with the remnant nuclei to about the same extent. This association does not appear to depend on the presence of chromatin and exists in addition to any possible association of hnRNP with chromatin itself.

Electron microscopy of partially purified nuclear hnRNA complexes shows that the hnRNP fibers form a ribonucleoprotein network throughout the nucleus, whose integrity is dependent on the RNA. Autoradiography confirms that hnRNA is a constituent of the fibers. The RNA network visualized in these remnant nuclei may be similar to RNA networks seen in intact cells.

The hnRNA molecules appear to be associated with the nuclear lamina, at least in part, by unusual hnRNA sequences. More than half of the recovered poly(A) and double-stranded hnRNA regions remains associated with the nuclear structures or the laminae after digestion with RNase and elution with 0.4 M ammonium sulfate. In contrast, the majority of oligo(A), another ribonuclease resistant segment, is released together with most of the partially digested but still acid-precipitable single-stranded hnRNA and the hnRNP proteins not eluted by the ammonium sulfate alone. These special RNA regions appear to be tightly bound and may serve as points of attachment of the hnRNA to nuclear substructures. It is suggested that hnRNA metabolism does not take place in a soluble nucleoplasmic compartment but on organized structures firmly bound to the nuclear structure.

KEY WORDS hnRNA · hnRNP fibers · nuclear lamina · double strands · poly(A)

This report describes the architecture of heterogeneous nuclear RNA-protein (hnRNP) within the cell nucleus. Fibers of hnRNP form a network within the nucleus which remains after the almost complete removal of chromatin. Special segments of the hnRNA (double-stranded regions and poly(A)) appear to serve as points of attachment to the nuclear substructure.

hnRNA molecules are a major transcriptional

product. Their metabolism (kinetic behavior) is complex (16) and they serve, at least in part, as precursors to cytoplasmic messenger RNA (for reviews, see references 17 and 18). The hnRNA molecules are known to be intimately associated with proteins in structures that have been visualized as ribonucleoprotein particles (hnRNP particles) by a number of investigators (1, 2, 5, 19, 22, 25, 26). The arrangement of hnRNP particles into a higher-order structural organization has been suggested by electron microscope investigations (2, 8, 9, 20, 27). These studies have revealed the presence of RNA fibers extending throughout the nucleoplasm, although the identification of the RNA in the fibers as hnRNA has been uncertain. The subnuclear fractionation procedure described here permits a biochemical study of the hnRNP fiber network.

MATERIALS AND METHODS

Cell Culture

HeLa S3 cells were grown in suspension culture at 37°C in Eagle's minimal medium supplemented with 7% horse serum. Density was maintained at 4×10^5 cells/ml. Where indicated, cells were labeled overnight with [^{14}C]thymidine (New England Nuclear, Boston, Mass.) (0.1 μCi/ml). Before labeling the hnRNA, the cells were concentrated fivefold and treated with actinomycin D (0.04 μg/ml) for 30 min to inhibit ribosomal RNA synthesis (24). [^{3}H]uridine or [^{3}H]adenosine (New England Nuclear) (50 μCi/ml) was added to the medium and the incubation was continued for 3 h or as otherwise indicated in the text.

Preparation of the Nuclear Lamina

Cells were washed with Earle's saline and resuspended in cold RSB (0.01 M NaCl, 0.01 M Tris pH 7.4, 1.5 mM $MgCl_2$). NP40 (Shell Chemical Co., Houston, Tex.) was added to a final concentration of 1%, and the cells were disrupted by vortexing. Nuclei were pelleted at 600 rpm and washed once in the same buffer. They were then resuspended in RSB supplemented to 0.11 M NaCl and divided into several equal portions. One portion was maintained on ice as a control. Electrophoretically purified DNase I (Worthington Biochemical Corp., Freehold, N.J.) was added (120 μg/ml) alone or in combination with RNase A (10 μg/ml) plus RNase T_1 (10 units/ml). Incubation was carried out at 25°C for 10 min. When the remnant cytoskeleton was to be removed, the double detergent (23) containing Tween 40 (Atlas Chemical Industries, Inc., Wilmington, Del.) and sodium deoxycholate was added. After vortexing, the nuclei were pelleted at 800 *g* for 3 min. The supernate has been designated the "released" fraction. The nuclear pellet was resuspended in 1 ml of 0.4 M $(NH_4)_2SO_4$, 30 mM Tris, pH 7.4. The laminae were then pelleted at 10,000 *g* for 15 min. Acid-precipitable radioactivity was determined for each fraction.

Distribution of Poly(A), Oligo(A), and Double-Stranded RNA

The distribution of poly(A) was determined by using cells labeled with [^{3}H]adenosine, and the distribution of double-stranded RNA was determined by using cells labeled with [^{3}H]uridine. After enzyme treatment and fractionation, as outlined above, the fractions of both control and digested nuclei were deproteinated by the phenol-chloroform procedure (28). RNA was recovered by ethanol precipitation and resuspended in HRSB (0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.01 M $MgCl_2$) and digested with DNase I (40 μg/ml) for 15 min at 37°C. The NaCl concentration was then increased to 0.25 M and RNase A (2.5 μg/ml) and T_1 (35 U/ml) were added. After 30 min at 37°C, the digestion was terminated by the addition of 0.5% sodium dodecyl sulfate (SDS). The resistant RNA was ethanol-precipitated with carrier yeast tRNA (100 μg/ml). Poly(A) was further purified by chromatography on oligo(dT)-cellulose (28) and then separated from nuclear oligo(A) by electrophoresis on 10% polyacrylamide gels as previously described (12). Poly(A) and oligo(A) distributions were obtained by summing the counts per minute migrating as poly(A) on the gels and expressing the amount present in each nuclear fraction as a percentage of the total poly(A) and oligo(A) recovered. Double-stranded RNA was separated from small oligonucleotides by electrophoresis on 15% polyacrylamide gels (14). The counts were summed and expressed as a percentage of the total recovered.

Electron Microscopy and Autoradiography

Samples were fixed, stained, and sectioned, as described previously (15). For autoradiography, the grids were coated with Ilford f4 emulsion and exposed for the times indicated in the legends to the figures.

SDS-Polyacrylamide Gel Electrophoresis

The nuclear laminae were prepared, as described above. The pellet was resuspended in SDS buffer (0.1 M NaCl, 0.01 M Tris pH 7.4, 5 mM EDTA, 0.5% SDS) and the proteins were precipitated with 10% TCA. The precipitate was washed once with cold absolute ethanol and resuspended in sample buffer (0.05 M Tris pH 6.8, 1% SDS, 0.02 M EDTA, 1% β-mercaptoethanol, 1% bromphenol blue, 10% glycerol) and boiled for 2 min before electrophoresis on a linear 10-15% polyacrylamide gradient SDS gel with a 5% stacking gel (29). A crude preparation of marker hnRNP proteins was prepared by a modification of the procedure of Pederson (22).

RESULTS

Association of hnRNA with the Nuclear Structure Remaining after Removal of Chromatin

The first step in these studies is the preparation of cellular nuclei using the non-ionic detergent NP40 and hypotonic buffer (RSB) (23). The complete removal of the nuclear DNA or chromatin requires a further step. As shown in Table I A, extensive digestion with pancreatic DNase I at neutral pH in the presence of Mg^{++} removes only part of the DNA. Presumably the resistant fraction is protected by its association with histones and nonhistone chromosomal proteins (6). However, most of this remaining DNA can be removed from the nuclei by raising the ionic strength with ammonium sulfate. Table I A shows that this procedure removes about 96% of the DNA from the nucleus, whereas over half of the hnRNA is retained. This result indicates that the retention of much of the hnRNA in the nucleus does not require most of the chromatin. This retained hnRNA appears associated with or anchored to the nuclear matrix or lamina.

The data in Table I A show that the bulk of steady-state hnRNA (labeled for 3 h) remains associated with the nuclear fraction after the removal of chromatin with DNase and ammonium sulfate. A different result might be expected for very short pulse-labeled hnRNA that is still nascent on its chromatin template. This new hnRNA might not yet have associated with the nuclear substructure. This is not the case, and the association of hnRNA with the nuclear structure occurs very quickly. The data in Table I B show that hnRNA pulse-labeled for very short periods (15 s) becomes associated with the nuclear ultrastructure remaining after the digestion and elution of the chromatin and, thus, that association must occur while the molecules are still nascent. The

TABLE I
Distribution of hnRNA

(A) Steady-state RNA (% of initial value before incubation)*

Fraction	Control		+DNase	
	hnRNA	DNA	hnRNA	DNA
	%		%	
Released from nucleus	4	0.2	4	4
Released in $(NH_4)_2SO_4$	2	0.0	20	53
Lamina	94	98.8	54	4
Lamina after 2nd digestion			37	1

(B) Pulse-labeled RNA (% of recovered RNA)‡

Fraction	15 s	45 s	90 s	150 s	210 s	3 h
Released from nucleus	17	20	15	20	20	19
Released in $(NH_4)_2SO_4$	17	20	12	13	13	18
Lamina	66	60	73	67	67	63

(C) Exogenous RNA (% of recovered RNA)§

Fraction	Control	+DNase
	%	
Released from nucleus	83	77
Released in $(NH_4)_2SO_4$	10	13
Lamina	7	10

* Cells were labeled with [^{14}C]thymidine (0.1 μCi/ml) for 16 h and then for 3 h with [^{3}H]uridine (50 μCi/ml). Acid-insoluble radioactivity in each fraction was determined.

‡ Cells were pulse-labeled for indicated times with [^{3}H]uridine (50 μCi/ml) and acid-insoluble radioactivity in each fraction was determined.

§ [^{3}H]uridine-labeled deproteinized hnRNA was added to unlabeled nuclei, and the distribution of acid-insoluble radioactivity was determined.

organization of hnRNA into structures appears to be an early event in the metabolism of these molecules.

The experiments presented so far show that hnRNP remains associated with the nuclear substructure after the removal of most of the chromatin. This suggests that the hnRNP is associated with nonchromatin structures within the nucleus or its lamina. However, 4% of the cellular chromatin is still in these preparations and the possibility that this material is both the site of attachment of hnRNP and uniquely resistant to DNase cannot be absolutely ruled out. This question is partly addressed by removing still more of the chromatin by a second round of digestion with DNAse. Apparently the proteins protecting the remant 4% of chromatin during the first digestion are either eluted or perturbed in their association with DNA by the first exposure to ammonium sulfate so as to render the protected DNA accessible during a second digestion. The result of a second nuclease treatment is also shown in Table I. Remnant chromatin has been reduced to 1%, whereas almost 40% of the hnRNA remains. This result further strengthens the suggestion that hnRNP has attachment sites in the nucleus, in addition to any possible association with chromatin. However, the binding of hnRNP to a nonchromatin nuclear substructure must remain putative until the binding sites are further characterized.

The association of hnRNP with the remnant nuclear structure probably reflects the actual state of this material in intact cells and does not result simply from an artifact of isolation. Nonspecific association through electrostatic interactions seems unlikely in view of the very high ionic strength used to prepare the chromatin-depleted nucleus. A nonspecific association of the hnRNA molecules themselves with the nuclear skeleton can be ruled out by mixing experiments. Deproteinated, labeled hnRNA is added to isolated unlabeled nuclei in amounts equivalent to 1/6 of the endogenous hnRNA. As shown in Table I C, very little of the exogenous hnRNA becomes associated with the nuclear ultrastructure of either intact or chromatin-depleted nuclei. A similar experiment using the lamina-associated hnRNP instead of hnRNA is difficult to envision, inasmuch as this material must first be removed from the nucleus, a step which would require extensive disruption of the hnRNP structures. The tightness of binding of the hnRNP to the remnant nucleus which resists high ammonium sulfate concentrations is the best indication of the absence of nonspecific association.

Morphology of the hnRNP Nuclear Fraction

The stepwise preparation of the hnRNP-containing nuclear fraction is examined next. Fig. 1 is an electron micrograph of a glutaraldehyde-fixed section of a HeLa cell nucleus. Both the cytoplasm and the nuclear membranes, together with the cytoskeleton, have been removed by detergent treatment (23). Nevertheless, an intact nuclear shell or lamina remains. Within this shell are prominent nucleoli and a network of nucleoprotein fibers containing both RNA and DNA. The nuclear chromatin is evenly distributed as a result of the low ionic strength used to prepare nuclei.

The hnRNP fibers can be visualized in Fig. 2, which is an electron micrograph of a nuclear preparation digested with DNase and eluted with

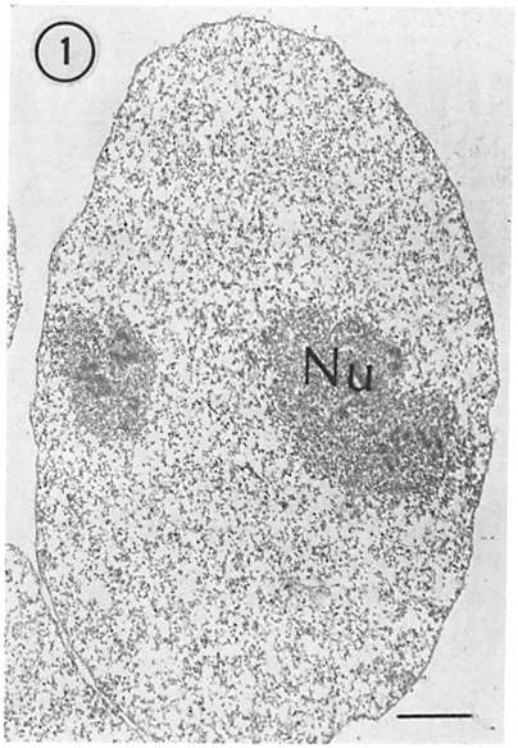

FIGURE 1 Thin section of HeLa cell nucleus. Nuclei were isolated as described in Materials and Methods and washed with the double detergent to remove the membranes (23). Fixation and staining was done as in reference 15. Dense nucleoli (*Nu*) are visible. Bar, 1 μm; × 9,000.

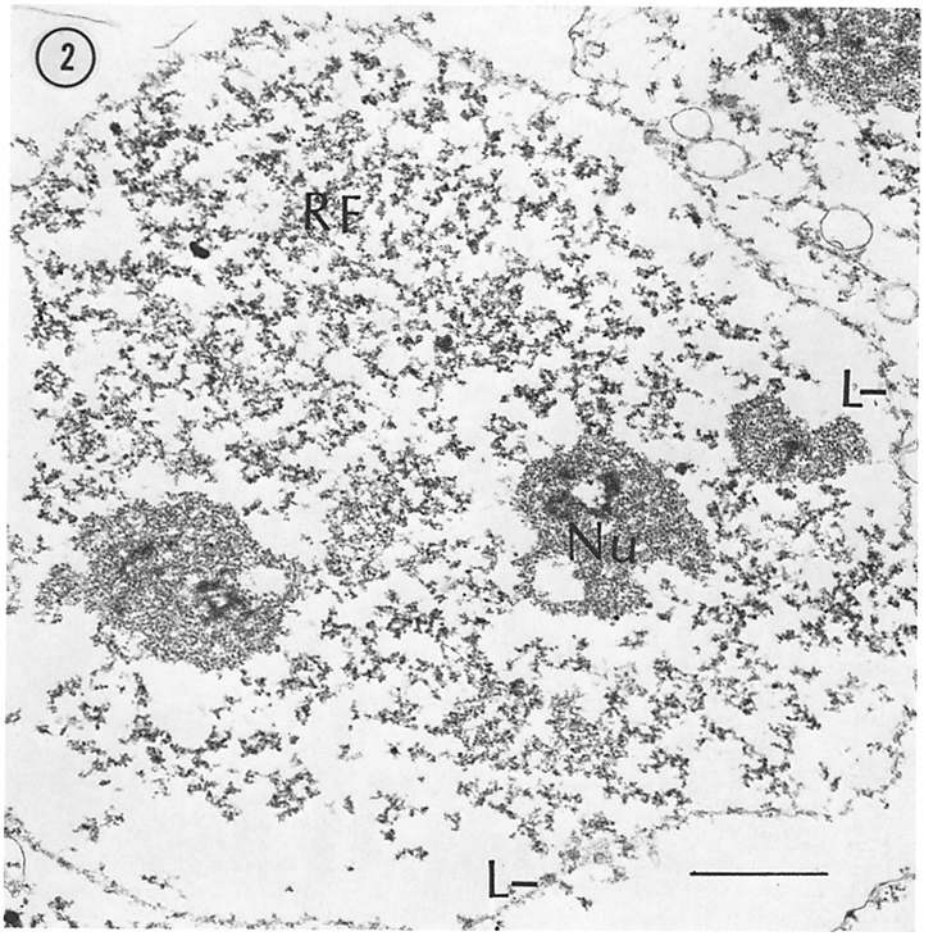

FIGURE 2 DNase-digested nucleus. Isolated nuclei were digested with DNase I (120 μg/ml) in RSB (0.11 M NaCl) for 10 min at 25°C. After washing with the double detergent and eluting with ammonium sulfate, the nuclei were pelleted at 3,000 *g* and prepared for electron microscopy. Intact nuclear lamina (*L*) contains nucleoli (*Nu*) and ribonucleoprotein fibers (*RF*). Bar, 1 μm; × 12,000.

ammonium sulfate. The lamina remains intact despite the nearly complete removal of the chromatin DNA. Nucleoli and an array of fibers comprised of RNA, remnant DNA, and protein are visible inside the lamina. The integrity and retention of these fibers depend on their RNA content. Fig. 3 shows the nearly empty lamina obtained after nuclei have been exposed to both RNase and DNase before ammonium sulfate elution. The RNA-containing network has been destroyed, leaving only an almost empty nuclear shell. Occasionally a densely staining cluster is seen inside the lamina (Fig. 3). These may be remnants of nucleoli but they cannot be identified with certainty.

We have assumed that the location of the RNA-containing fibers in the extranucleolar space or nucleoplasm indicates that hnRNA is the principal RNA component. However, it could be argued that the RNA-containing fibers may be composed of (unfolded) ribosomal subunits en route from the nucleolus to the cytoplasm.

Electron microscope autoradiography after selective labeling of hnRNA shows the occurrence of hnRNA in the nuclear RNA fiber network. An exponentially growing culture of HeLa cells is pretreated for 30 min with a low concentration of actinomycin D to inhibit selectively the transcription of the ribosomal genes (24) before the addition of [^{3}H]uridine to the medium. The cells are then incubated for additional 2.5 h to allow an approach to steady-state labeling. Nuclei, isolated

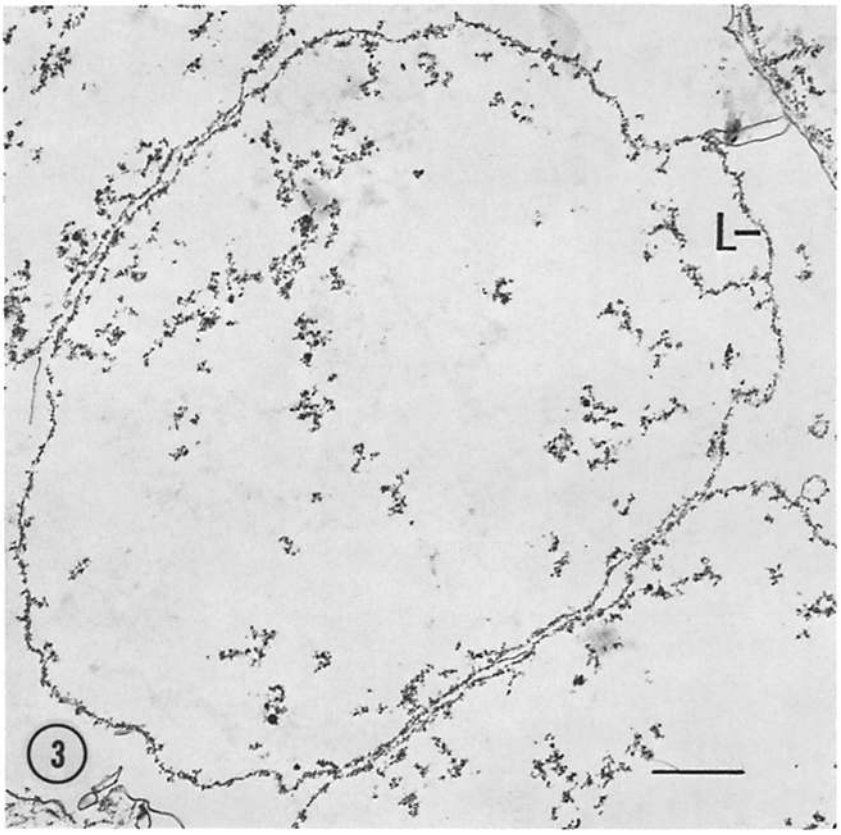

FIGURE 3 DNase- and RNase-digested nucleus. A portion of the nuclear preparation (Fig. 2) was digested with DNase I (120 μg/ml) plus RNase (10 μg/ml) and RNase T_1 (10 U/ml) for 10 min at 25°C before detergent and ammonium sulfate treatment. The lamina (*L*) remains intact but the majority of the fibers have been removed. Bar, 1 μm; × 12,000.

as outlined in Materials and Methods, were then digested with enzymes, washed, and eluted with ammonium sulfate. After preparation for electron microscopy, the grids were coated with photographic emulsion and exposed for the times indicated in the figure legends.

Fig. 4 is an autoradiograph of the nuclear preparation, selectively labeled for hnRNA and treated with DNase and ammonium sulfate. Numerous silver grains are seen in the regions containing the RNA fibers. As expected, no grains are detected over the nucleolus because of the selective inhibition of nucleolar transcription by actinomycin. Regions not containing fibers also do not contribute a significant number of silver grains.

Fig. 5 is an autoradiograph of a portion of the same nuclear preparation that was treated with both DNase and RNase under conditions which released >90% of the incorporated ^3H counts per minute. It is apparent that the majority of both the fibers and the silver grains have been eliminated by RNase digestion. This confirms that the radioactive precursor was incorporated into RNA and supports the conclusion that these fibers contain hnRNA. It is likely that most of the remaining fibers contain the residual DNA which resists elution by ammonium sulfate.

Association of Unusual hnRNA Sequences with Subnuclear Structure

The tight association of hnRNP fibers with the remnant nuclei after the removal of chromatin suggests an attachment of the fibers to subnuclear structure or perhaps to the nuclear lamina itself. The RNA-containing fibers seen in the electron micrographs are distributed throughout the nuclear space, and some appear to associate with or

terminate on the nuclear lamina. The bulk of the hnRNA is removed as large fragments by digestion with RNase, but significant amounts of unusual hnRNA sequences such as the poly(A) (21) and double-stranded regions (14) remain behind, as shown by the following measurements. These segments may serve as regions of attachment of the hnRNP fibers.

The tightly bound, RNase-resistant regions of hnRNA are studied by disrupting the integrity of the hnRNP fibers in the nuclear preparation with RNase and eluting the remaining fragments with ammonium sulfate. At least half of the hnRNA is protected from nuclease attack by virtue of its associated proteins, but it is now largely removed by exposure to high ionic strength (see Figs. 3 and 5). Only 2% of the original hnRNA remains acid-precipitable and attached to the lamina after the enzyme digestion and elution with ammonium sulfate. In contrast, data in Table II A show that more than half of the recovered poly(A) remains attached to the nuclear ghost despite the nearly complete removal of the hnRNA. The poly(A) is associated with the nuclear lamina in an EDTA-sensitive linkage and thus can be eluted from it after digestion by pancreatic ribonuclease by the addition of 0.01 M EDTA (Table II A). The eluted poly(A) is obtained in the form of a particle, presumably complexed with protein, as evidenced by its sedimentation value (12S) and buoyant density in low ionic strength sucrose and metrizamide gradients (not shown). This poly(A)-containing particle is similar to that previously described by other investigators (25, 26). An association of the poly(A) particle with the nuclear substructure or lamina is suggested. The fact that the poly(A) particles resist elution of 0.4 M ammonium sulfate implies, however, that this

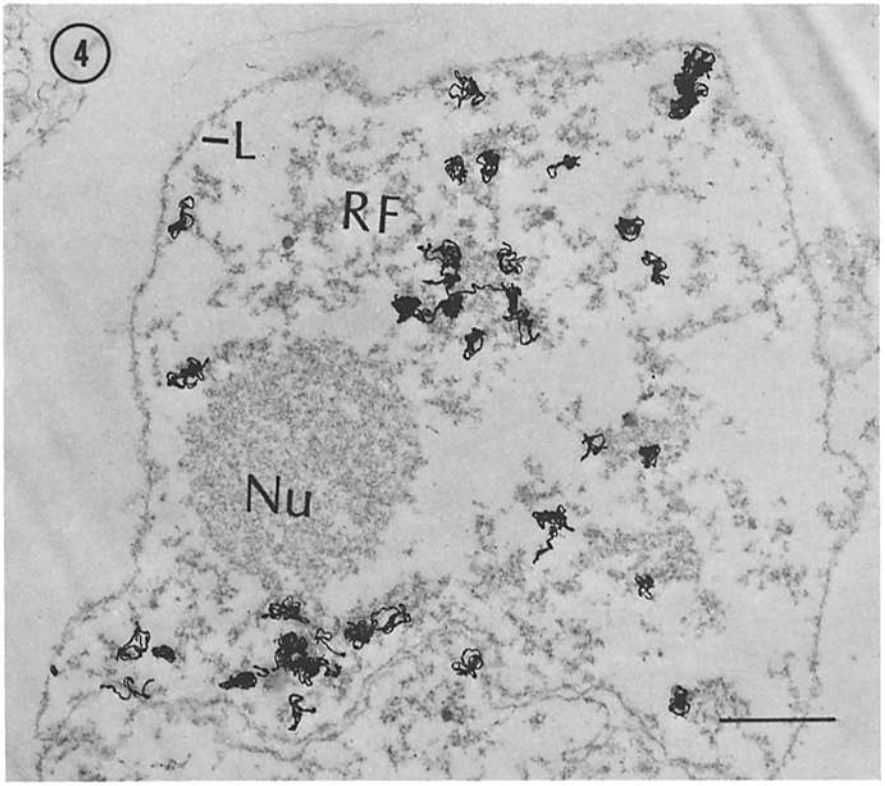

FIGURE 4 Autoradiograph of DNase-digested nucleus. Cells were concentrated fivefold and treated with actinomycin D (0.04 μg/ml) for 30 min. [^{3}H]uridine was then added to 10 μCi/ml and incubation continued for 2.5 h. The nuclei were isolated as described in Materials and Methods. DNase-digested nuclei were detergent-washed and ammonium-sulfate-eluted, as described in the legend to Fig. 2. Grids were coated with Ilford f4 emulsion and exposed for 21 days. Symbols are defined as in the legend to Fig. 2. Bar, 1 μm; × 15,000.

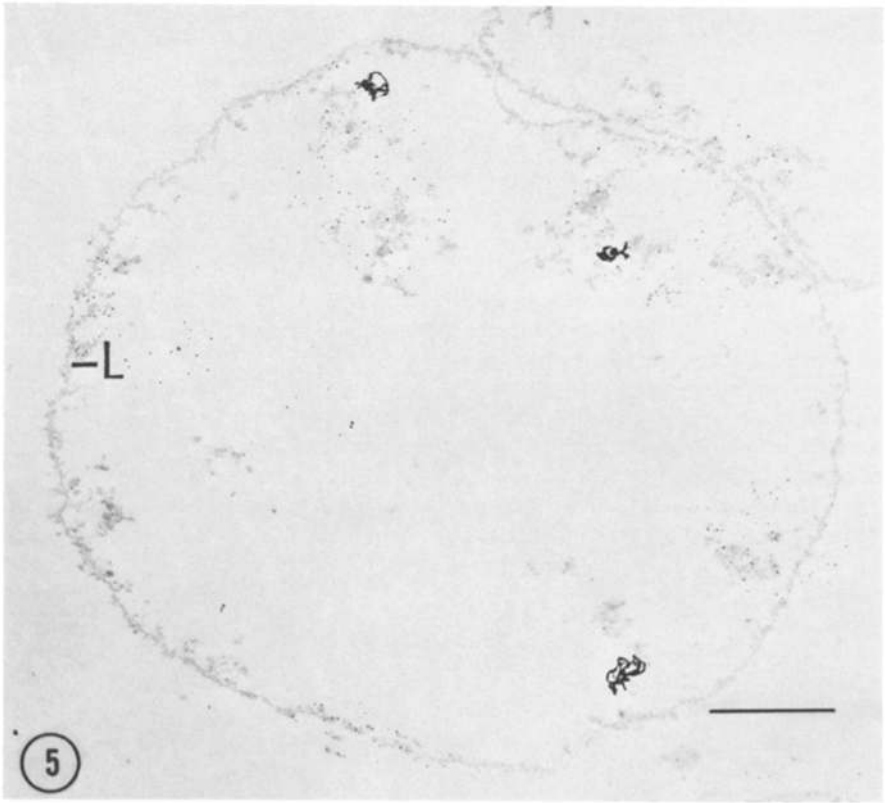

FIGURE 5 Autoradiograph of DNase- and RNase-digested nucleus. A portion of the labeled nuclei (Fig. 4) was digested with both DNase and RNase, as described in the legend to Fig. 3, before detergent and ammonium sulfate treatment. The grids were coated with emulsion and exposed for 28 days. Symbols as in Fig. 3. Bar, 1 μm; × 14,400.

association is not simply adventitious.

The other class of hnRNA segments that remain attached to a significant degree to the nucleus is the double-stranded regions of the hnRNA. Table II B shows that 56% of the double-stranded regions in hnRNA, recovered after ribonuclease digestion of intact nuclei, is associated with the nuclear lamina after elution with ammonium sulfate even though only ~2% of the original hnRNA remains in this fraction. In contrast to the result with poly(A), association of double-stranded RNA with the nucleus is not altered by the presence of EDTA (not shown).

The retention of poly(A) and double-stranded regions by the remnant nuclei after ribonuclease digestion is not simply a reflection of their ribonuclease resistance. First, and most important, the released RNA measured here is only that material which remains acid-precipitable, presumably by virtue of protection by hnRNP proteins. Thus, of the protein protected, nuclease-resistant RNA, most is released as compared to the double-strand and poly(A) segments. Second, another intrinsically ribonuclease-resistant segment, the internal oligo(A) of hnRNA, behaves very differently from poly(A) and double-stranded regions. Table II shows that oligo(A) is largely released after ribonuclease digestion and elution with ammonium sulfate.

It should be noted that approximately a twofold increase in the content of double-stranded regions is observed after the deproteinization of HeLa hnRNP.[1] This apparently reflects the development of double-stranded regions in hnRNA sequences after the removal of proteins, and these developed double strands are not native. Therefore, it is not unexpected that the hnRNA digested in isolated

[1] T. Pederson, personal communication.

nuclei contrains less (43%) double-stranded RNA than does control hnRNA (Table II B).

Release of hnRNP Proteins by RNase

A spectrum of proteins has been shown to be associated with hnRNP with three major polypeptides having molecular weights of 32,000, 34,000, and 63,000 daltons (5). The 32,000-dalton protein appears to be more tightly bound than the other two and remains attached after exposure to ammonium sulfate. Fig. 6 shows an SDS-polyacrylamide gel of Coomassie-Blue-stained nuclear proteins. Fig. 6*a* shows the proteins present in the nuclear lamina preparation after DNase digestion and ammonium sulfate elution. The histones are almost completely removed as are the 34,000- and 63,000-dalton hnRNP proteins. However, a significant amount of the 32,000-dalton protein remains, as well as many other hnRNP proteins. For comparison, Fig. 6*b* shows the proteins present in a preparation of hnRNP particles. When the nuclei are digested with RNase and DNase before ammonium sulfate treatment, almost all of the remaining hnRNP protein is removed, as shown in Fig. 6*c*. Of the remaining 30–40 bands, the most prominent are in the molecular weight range of 40,000–70,000 daltons and possibly represent the structural proteins of the lamina (3, 13). Thus, the retention of much of the hnRNP protein in these nuclear preparations requires the integrity of hnRNA. This implies a limited number of attachment sites for hnRNP fibers in the nuclear preparations described.

TABLE II

Associations with Nuclear Lamina

(A) Distribution of poly(A)*

	Control	+DNase +RNase	+DNase +RNase +EDTA
	%	%	%
Percent recovery	100	88	84
Released from nucleus	4	29	71
Released in $(NH_4)_2SO_4$	15	16	12
Lamina	81	55‡	17

(B) Distribution of double-stranded RNA‡

	Control	+DNase§ +RNase
	%	%
Percent recovery	100	43
Released from nucleus	5	13
Released in $(NH_4)_2SO_4$	6	31
Lamina	89	56‡

(C) Distribution of oligo(A)

	Control	+DNase +RNase
	%	%
Percent recovery	100	70
Released from nucleus	7	36
Released in $(NH_4)_2SO_4$	2	48
Lamina	91	16

* Cells were labeled for 3 h with [^{3}H]adenosine (50 μCi/ml) and fractionated as indicated. After deproteinization, each fraction was redigested (see Materials and Methods) and the poly(A) and oligo(A) were isolated by chromatography on oligo(dT)-cellulose. Poly(A) and oligo(A) were resolved by electrophoresis on 10% polyacrylamide gels. Radioactivity across each gel peak was summed and expressed as a percentage of the total poly(A) or oligo(A).

‡ Cells were labeled for 3 h with [^{3}H]uridine (50 μCi/ml) before fractionation. Each fraction was deproteinized and redigested as described in Materials and Methods. Resistant RNA was resolved on 15% polyacrylamide gels, and radioactivity in double-stranded RNA was summed across each gel.

§ After this digestion, the lamina contained only 2% of the original hnRNA.

DISCUSSION

These experiments suggest that the hnRNA in the HeLa cell nucleus can be visualized as a network of RNA- and protein-containing fibers in the HeLa cell nucleus after removal of 96–99% of chromatin with DNase and ammonium sulfate. This network is presumably related to that seen in electron micrographs in which selective bleaching of chromatin has been used to enhance the visibility of the RNA-containing nuclear fibers (4). Newly synthesized hnRNA becomes associated immediately with the nonchromatin nuclear structure to about the same extent as steady-state RNA.

~98% of the hnRNA can be removed from the nuclei by digestion with pancreatic DNase and RNase and elution with ammonium sulfate. The remnant RNA is highly enriched in unusual sequences, which suggests that these may play a role in the attachment of hnRNA to the nuclear ultrastructure. Over half of the recovered poly(A) remains associated with the chromatin-depleted nuclear preparation in an EDTA-sensitive linkage. This association resists elution by ammonium

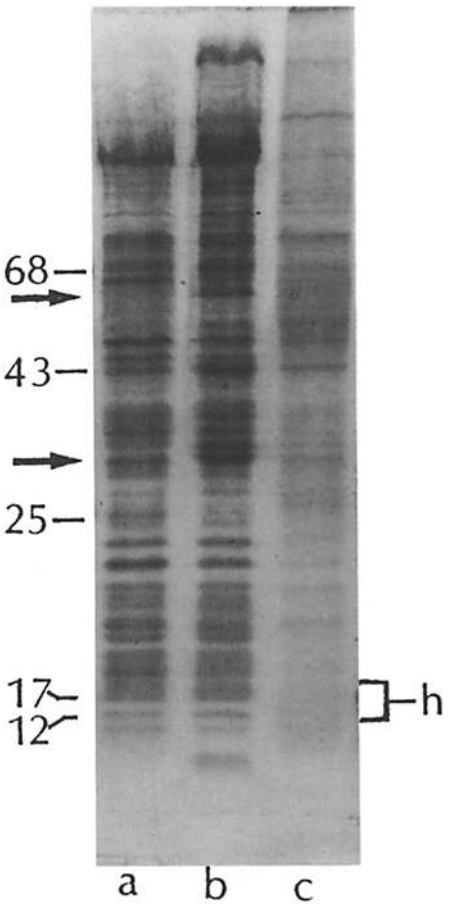

FIGURE 6 Gel electrophoresis of lamina proteins. Nuclei were prepared and enzyme digested as described in Materials and Methods. After detergent treatment and ammonium sulfate elution, the proteins were prepared for electrophoresis as described in Materials and Methods. Electrophoresis was done on a 10–15% polyacrylamide SDS gel at 120 V for 4.5 h (29). The gel was fixed and stained in 50% methanol, 7% acetic acid, 0.2% Coomassie Blue. The numbers indicate the positions of protein molecular weight standards run in a parallel lane: 68,000, bovine serum albumin; 43,000, ovalbumin; 25,000, chymotrypsinogen; 17,000, myoglobin; 12,000, cytochrome c. The arrows indicate the positions of the 32,000-, 34,000-, and 63,000-dalton hnRNP proteins. (*a*) DNase-digested; (*b*) crude hnRNP protein preparation; (*c*) DNase- and RNase-digested. The position where the histones migrate is marked (*h*). Each lane contains the proteins isolated from 1×10^7 nuclei.

sulfate and may represent the in vivo association of a significant fraction of the poly(A) with the nuclear framework. In addition, ~50% of the native double-stranded nuclear RNA remains attached to the nuclear structure. This suggests that double-stranded RNA regions may be a component of the sites which anchor the fibers to the nuclear substructure or lamina.

The hnRNP fibers described in this report appear attached to nuclear substructure sites in addition to any possible chromatin sites. The precise structural elements containing these sites is not revealed by either the morphological or biochemical studies. There appears to be little in the way of an internal protein matrix in these preparations and, yet, the putative attachment regions of hnRNA molecules (double-stranded and poly(A) segments) are extensively retained. One possible structure which may serve as an anchor for some of the hnRNP fibers is the nuclear lamina itself. Occasional contacts between the fibers and the lamina can be seen in Figs. 2 and 4. Alternatively, there may be other remnant structures in the nucleus, such as an internal matrix, which are either not visualized by or not preserved during the microscopy procedures employed here.

Conceivably, the remnant chromatin might serve as the sole anchoring site for the hnRNP fibers. The second digestion with DNase, followed by a second high-salt elution, indicates that this is not likely, though it remains a possibility.

Also, using an entirely different experimental approach, Faiferman and Pogo (8) recently suggested the occurence of hnRNA in a fibrillar network associated with the nuclear lamina.

The nuclear lamina preparations shown here after DNase and RNase treatment are rather empty and resemble those of Dwyer and Blobel (7) much more than they do the nuclear skeletons, with internal protein matrix, prepared from rat liver by Berezney and Coffey (3). The absence of the internal matrix described by these authors is not understood at the present time but may be due to differences either in cell type or in methods of preparation. The results presented here suggest that, apart from the nucleolus, most of the internal nuclear structure that remains after the removal of the chromatin in high salt is composed of hnRNA-containing fibers.

Recently, Hodge et al. (13) presented evidence for the existence of an internal nuclear protein matrix in HeLa cells. The procedure used by these authors to prepare the nuclear lamina exposed the isolated nuclei to high ionic strength before DNase digestion. Under these conditions, we have found that most of the nuclear laminae rupture as a

result of the unfolding of the chromatin (unpublished observation, Ronald Herman). It is likely that those that remain intact still contain some nucleic acids, and this may be the origin of the internal matrix observed by Hodge et al. The presence of histones in their preparation suggests that the observed matrix is composed, at least in part, of residual chromatin (13). Nevertheless, all methods so far described for visualizing the internal nuclear structure remove considerable amounts of material, and a complete analysis of the nuclear architecture must await resolution of the discrepancies in the results of different investigations.

The results presented here suggest an organized structure of hnRNA fibers which may play a role in the processing of these molecules. The necessity of an ordered structure for proper processing may explain some of the anomalous effects of actinomycin D on both RNA metabolism and DNA organization. In two distinct experimental systems, the normal processing of hnRNA has recently been found to be altered by the administration of a high concentration of actinomycin (16, 24). Actinomycin thus causes a prompt cessation of both RNA synthesis and processing. Continued RNA synthesis has also been shown to be a requirement for the proper processing of precursor rRNA in the nucleolus (10). The correct processing of hnRNA may, perhaps, require the continuation of RNA synthesis to prevent derangement of the hnRNA fiber network. Similarly, if the internal nuclear structure is comprised largely of RNA-containing fibers and if these play a role in chromatin arrangement, then it may be possible to understand the drastic changes in chromatin morphology which occur after the cessation of RNA synthesis (11). hnRNA may not only be the precursor to cytoplasmic mRNA but may also serve as a component of nuclear architecture.

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