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Biochemistry

Nuclear ribonucleoprotein particles probed in living cells*

(heterogeneous nuclear RNA·protein complexes/photochemical RNA–protein crosslinking *in vivo*/nuclease digestion/Cs_2SO_4 banding/mRNA processing and nuclear structure)

SANDRA MAYRAND AND THORU PEDERSON

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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ABSTRACT **Contacts between heterogeneous nuclear RNA (hnRNA) and protein in nuclear ribonucleoprotein particles have been photochemically crosslinked in intact HeLa or Friend erythroleukemia cells by irradiation with 254-nm light at doses of 10^1 to 10^5 ergs/mm^2 (1 to 10^4 μJ/mm^2). The resulting crosslinked particles were isolated and compared with conventional hnRNA·protein (hnRNP) preparations. By the criteria of nuclear fractionation behavior, sedimentation coefficients, nuclease digestion profiles, and RNA-to-protein ratio measured by banding in Cs_2SO_4 density gradients, the hnRNP particles crosslinked *in vivo* are identical to nonirradiated particles. Gel blot hybridization of RNA from Friend cell hnRNP crosslinked *in vivo* reveals that β-globin RNA sequences remain both intact and hybridizable after the irradiation procedure. The crosslinked hnRNA–protein bonds are stable in 8 M urea/0.5% sodium dodecyl sulfate and withstand centrifugation in Cs_2SO_4 gradients of initial density 1.50 g/cm^3. These results establish that hnRNA is tightly complexed with nuclear proteins *in vivo* and that hnRNP particles isolated by nuclear fractionation represent native structures.**

Most eukaryotic genes are interrupted by DNA that does not code for mRNA. These intervening DNA sequences are transcribed into heterogeneous nuclear RNA (hnRNA) colinearly with mRNA-coding regions and are then excised, and the mRNA segments are rejoined. Consequently, there has recently been a resurgence of interest in hnRNA → mRNA processing. We have been studying hnRNA·protein complexes, known as hnRNP particles (reviewed in ref. 1), because we suspect that this nucleoprotein structure of hnRNA may be an important aspect of its processing. The idea that hnRNA is associated with protein in the cell has its roots in the studies by Gall (2) and Callan and Lloyd (3) of nascent ribonucleoprotein (RNP) on the lateral loops of amphibian lampbrush chromosomes. More recently, the association of proteins with hnRNA has been further documented by ultrastructural analysis of nuclear RNP particles *in situ* (4–6) or of nascent, nonribosomal RNP fibers on chromatin spread by the technique developed by Miller (7–10).

Just as the reliability of these ultrastructural studies of hnRNP (4–10) depends on critical fixation and related aspects of specimen preparation, the biochemical isolation of hnRNP particles (11, 12) requires attention to the possibilities of macromolecular rearrangements and nonspecific RNA–protein associations during cell fractionation. We, and others, have addressed this issue through reconstruction experiments in which deproteinized hnRNA is added to nuclei prior to hnRNP particle isolation (12–15). The results have been reassuring in that the added hnRNA does not pick up significant amounts of protein, indicating the absence of a large nuclear pool of nonspecific RNA-binding proteins. Yet, the caveat in all such reconstructions is the possibility that nonspecific RNA-protein associations have already occurred during nuclear isolation so that, by the time the naked hnRNA probe is added, the reacting proteins have been depleted by the formation of what the observer (mistakenly) scores as "endogenous" hnRNP.

Because the current emphasis on hnRNA → mRNA processing is likely to trigger a parallel resurgence of interest in hnRNP, we have decided to re-examine the authenticity of isolated hnRNP particles by a different approach. We describe here the results of experiments in which hnRNA–protein associations are probed in living cells by photochemical RNA–protein crosslinking. This approach is based upon two specific attributes of RNA–protein crosslinking by 254-nm light (16–19). The first is the ability of UV light, at sufficient doses, to penetrate deeply into biological structure (e.g., intact cells). The second is the fact that the crosslinking radius of this probe is extremely short, so that only proteins in direct contact with the RNA are crosslinked (e.g., see ref. 20). These two features have made it possible for us to employ 254-nm light to assess the extent to which hnRNA–protein contacts exist in the intact cell prior to fractionation.

METHODS

Cells. HeLa (human) and Friend erythroleukemia (mouse) cells were grown as previously detailed (15, 21). hnRNA was selectively labeled by pulsing cells for 20 min with [^{3}H]uridine after selective suppression of ribosomal RNA synthesis by 0.04 μg of actinomycin per ml for HeLa cells (12) and 0.08 μg/ml for mouse cells (15). Labeling was terminated by pouring the cell suspension into ice-cold balanced salt solution (22) without phenol red. After centrifugation the cells were washed in balanced salt solution, then once again in buffered saline (0.15 M NaCl/10 mM Tris·HCl, pH 7.2) and then resuspended at 10^7 cells per ml in buffered saline for irradiation.

Photochemical Crosslinking. Suspensions, 20 ml, 10^7 cells per ml, were transferred to pre-chilled 100-mm petri dishes, lined on the inner surface with aluminum foil and kept at 4–6°C with stirring. (More recently we have used highly polished stainless steel petri dishes.) The UV irradiation source was a pair of 2.5 × 42 cm 15-W Sylvania germicidal lamps (G15-T8) mounted in parallel 1 cm apart and equipped with reflectors fabricated of aluminum foil. The incident intensity of 254-nm light was measured with a UV meter (model J-225, Ultra-Violet Products, San Gabriel, CA) in the plane of the surface of the cell suspension. Intensity was varied by adjusting the distance between the cell suspension and the UV source. The highest UV doses employed were 3.6 × 10^5 ergs/mm^2 (1 erg = 10^{-7}

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Abbreviations: hnRNA, heterogeneous nuclear RNA; RNP, ribonucleoprotein; hnRNP, hnRNA·protein particle; RSB, 10 mM NaCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.2.

* This is paper no. 14 in a series entitled "Ribonucleoprotein organization of eukaryotic RNA." Paper no. 13 is ref. 31.

J), which were obtained with a distance of 6.25 cm (flux = 4000 $\mu W/cm^2$) and an irradiation period of 15 min. Lower doses (Fig. 1) were obtained by reducing the duration of irradiation, the intensity, or both.

Isolation of hnRNP and Analysis of RNA–Protein Crosslinking. After irradiation, the cells were harvested and resuspended in RSB (10 mM NaCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.2) and fractionated as described for the isolation of hnRNP (12, 15, 23). In most of the experiments reported here, a postnucleolar fraction containing both chromatin and hnRNP was used, while in some cases hnRNP was purified on sucrose gradients as indicated.

Crosslinking was monitored by the phase partitioning of pulse-labeled hnRNA in a phenol/water system. To hnRNP from untreated or irradiated cells, NaCl was added to 0.1 M, EDTA to 0.02 M, urea to 8 M, and sodium dodecyl sulfate to 0.5%. After aliquots had been taken for determination of total trichloroacetic acid-precipitable ^3H radioactivity, the samples were extracted for 30 min at 20–22°C with 1 vol of buffered freshly prepared phenol/chloroform/isoamyl alcohol (50:49.5:0.5, vol/vol). After separation of the organic and aqueous phases by centrifugation, aliquots were taken from the aqueous phase for determination of acid-precipitable radioactivity. Crosslinking was computed as detailed in Fig. 1.

RESULTS

RNA–Protein Crosslinking of hnRNP Particles at Three Levels of Organization. A facile assay for photochemical RNA–protein crosslinking is the phase partitioning of RNA in a phenol/water solvent system (e.g., see ref. 17). In the absence of crosslinking, 85–90% of the [^3H]uridine pulse-labeled RNA in HeLa cell hnRNP particles partitions in the aqueous phase after phenol extraction. As shown in Fig. 1, after 254-nm irradiation a dose-dependent increase occurs in the fraction of hnRNA partitioning in the phenol phase. (Irradiation of deproteinized hnRNA alone does not cause it to become soluble in phenol solution.) This progressive shift of hnRNA into the phenol phase is observed despite the presence in the extraction buffer of 0.5% sodium dodecyl sulfate and 8 M urea. The addition of 0.1% 2-mercaptoethanol to the extraction buffer does not influence the extent of hnRNA-protein crosslinking, indicating that protein–protein disulfide bond formation does not contribute significantly to the results. It can be seen in Fig. 1 that sigmoidal dose–response curves are obtained for hnRNP irradiated as isolated particles, in purified nuclei, or in intact cells, and that higher doses are required with increasing biological complexity, as would be expected. In all cases, 80–90% of hnRNA can be crosslinked to protein, as defined by conferring solubility in phenol solution on the normally phenol-insoluble hnRNA. These results demonstrate that, at all three levels of organization, hnRNA is in direct contact with protein. For the case of intact cells, this result confirms the earlier cytological and ultrastructural observations that proteins are associated with hnRNA. It also serves as a point of departure for examining the extent to which particles that have been crosslinked *in vivo* resemble those normally isolated as "hnRNP." Therefore, for the remainder of this paper, all the experiments presented deal with hnRNP crosslinked *in vivo*.

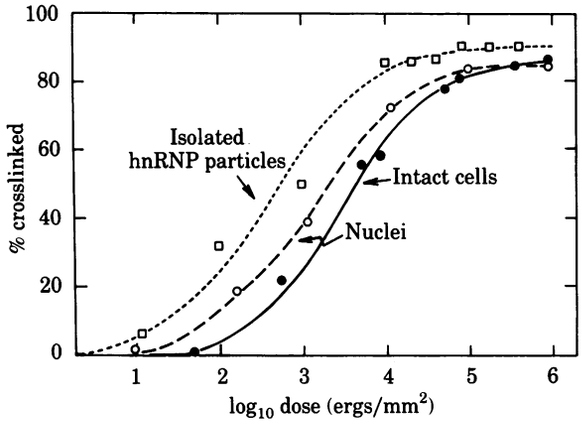

FIG. 1. hnRNA–protein crosslinking as a function of UV dose. HeLa cells were pulse labeled with [^3H]uridine in the presence of 0.04 μg of actinomycin per ml and irradiated at 10^7 cells per ml as detailed in *Methods*. In other experiments nuclei were isolated as previously described (12) and irradiated at 10^7 nuclei per ml in RSB. hnRNP particles were purified on sucrose gradients (12) and irradiated at an A_{260} of 0.04. Crosslinking was measured by phenol extraction in the presence of 8 M urea and 0.5% sodium dodecyl sulfate. The "% crosslinked" is $(x - y)/x$, in which x is the percentage of trichloroacetic acid-precipitable [^3H]uridine radioactivity in the aqueous phase of noncrosslinked hnRNP and y is the percentage of ^3H radioactivity in the aqueous phase of irradiated hnRNP. x was always between 85% and 90% of the total ^3H radioactivity, due to the partitioning of 10–15% of the hnRNA into the phenol phase even in the absence of crosslinking.

Specificity and Properties of Crosslinked hnRNP. Control experiments have been conducted to assess the specificity of crosslinking. Mixtures of deproteinized hnRNA and bovine serum albumin irradiated at 10^5 ergs/mm^2 were not detectably crosslinked, confirming the earlier results of Schoemaker and Schimmel for tRNA/bovine serum albumin mixtures (17). Deproteinized [^3H]hnRNA irradiated at 3.6×10^5 ergs/mm^2 in the presence of a 1000-fold mass excess of HeLa nuclear protein was crosslinked with some protein, but these complexes had a much lower protein-to-RNA ratio than *bona fide* hnRNP particles as measured by their increased buoyant density in Cs_2SO_4 (ρ = 1.45–1.50 g/cm^3) relative to native hnRNP (ρ = 1.30 g/cm^3). In addition, the hnRNA–protein complexes formed *in vitro* had a much higher sensitivity to pancreatic RNase digestion than native hnRNP did. In particular, these hnRNA–protein complexes were 70% digested under conditions that produced 80% digestion of naked hnRNA, but only 50% digestion of endogenous hnRNP. Thus, while there appears to be some interaction between added hnRNA and nuclear protein, at least enough to be stabilized by photochemical crosslinking, the resulting complexes do not resemble endogenous hnRNP particles (see also ref. 15).

Despite the photochemically catalyzed formation of covalent hnRNA–protein bonds (Fig. 1), the physical properties of the crosslinked hnRNP particles do not appear to be significantly altered. The nuclear fractionation behavior and recovery of hnRNP is unaffected by crosslinking. The percentage of total nuclear [^3H]uridine radioactivity recovered in the postnucleolar supernatant of control and irradiated cells was 84.2% and 79.3%, respectively (averages of seven determinations each). In addition, sucrose gradient analysis revealed the sedimentation properties and recoveries of control and crosslinked hnRNP particles to be indistinguishable (data not shown). In experiments probing the accessibility of hnRNA in the particles to pancreatic RNase, there was no detectable difference in the digestion profiles of control and crosslinked hnRNP (Fig. 2). It can be seen in Fig. 2*A* that the degree of RNase protection afforded by hnRNP proteins, relative to naked hnRNA, is exactly the same for control and crosslinked particles. In Fig. 2*B*, the rate of hnRNP digestion has been accelerated by increasing the RNase-to-hnRNP ratio, allowing the digestion kinetics of control and crosslinked particles to be followed over a range in which approximately 75% of the hnRNA is under analysis. As in Fig. 2*A*, there is no difference between control and cross-

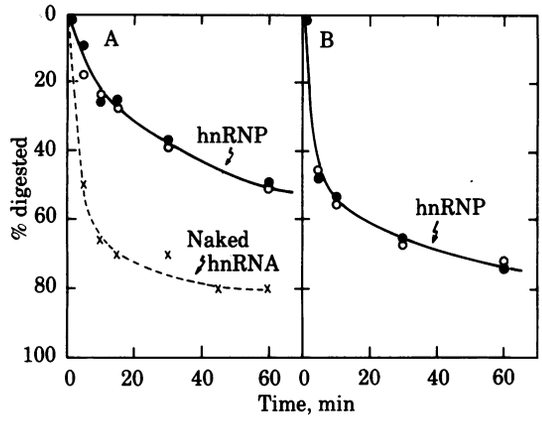

FIG. 2. Similarity of crosslinked and noncrosslinked hnRNP particles with respect to nuclease accessibility. hnRNP particles in RSB were digested at 4°C with pancreatic RNase at a concentration of 0.1 μg/ml (*A*) or 1.0 μg/ml (*B*). Digestion was monitored by the conversion of [^{3}H]uridine radioactivity to trichloroacetic acid-soluble form. ●, hnRNP crosslinked *in vivo*; ○, noncrosslinked particles; x, deproteinized [^{3}H]hnRNA.

linked hnRNP. Results similar to those in Fig. 2 were obtained with hnRNP from Friend erythroleukemia cells crosslinked *in vivo* (not shown). These results demonstrate that, by the criterion of nuclease protection, *in vivo* crosslinking does not capture a significant amount of additional nuclear protein beyond that normally isolated with hnRNA as hnRNP.

Effects of Crosslinking on an hnRNP Particle-Bound Enzyme. In addition to probing the structure of hnRNP by pancreatic RNase digestion (Fig. 2), it was of interest to examine the effects of crosslinking on an endogenous ribonuclease activity that cofractionates with hnRNP particles (ref. 24 and unpublished results). As shown in Fig. 3*A*, when control hnRNP particles are recovered from sucrose gradients and incubated at 37°C in 10 mM NaCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.2, about 22% of the particle-associated hnRNA is digested to acid solubility over a 2-hr period. (No digestion occurs in particles allowed to stand at 4°C.) As shown by the open circles in Fig. 3*A*, the kinetics of this "self-digestion" of hnRNP are not detectably altered when the concentration of hnRNP is reduced to 1/10th, indicating that the observed digestion is an intraparticle event and suggesting therefore that the responsible nuclease(s) is particle bound. Fig. 3*B* shows the results of a comparable experiment with hnRNP crosslinked *in vivo*. It can be seen that the kinetics of the reaction are similar to that of control particles, reaching a nearly identical value of 20% digested at 2 hr. Once again, a reduction to 1/10th in hnRNP particle concentration does not alter the reaction kinetics. These results therefore show that a presumptively particle-bound enzyme(s) is not inactivated by the irradiation conditions used to crosslink the hnRNP. These data also suggest that the endogenous nuclease has equal accessibility to the hnRNA in control and crosslinked hnRNP. We emphasize that these results do not address the question of whether or not the hnRNP nuclease is crosslinked to the hnRNA. Rather, the results simply provide another criterion by which the control and crosslinked particles are indistinguishable.

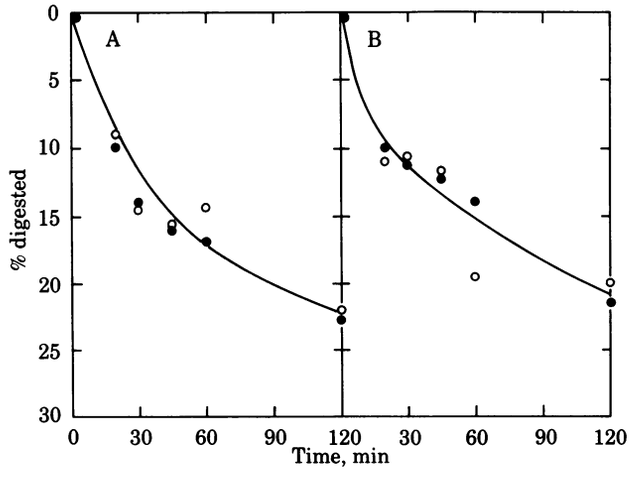

FIG. 3. Activity of an hnRNP-associated nuclease. hnRNP particles were purified on sucrose gradients in RSB (12) and incubated at 37°C to permit the action of an endogenous nuclease. (*A*) Results for noncrosslinked hnRNP; (*B*) particles crosslinked *in vivo*. ●, Particles at the concentration in which they are directly recovered from the gradients, which in these experiments corresponded to an A_{260} of 0.1. ○, Comparable data for particles that were diluted 1:10 with RSB before assay. Note that full scale on the ordinate is 30% digested.

Covalent Integrity of a Specific mRNA Sequence in Crosslinked hnRNP. To examine the important possibility of radiation-induced breakage of hnRNA in crosslinked particles, Friend erythroleukemia cells were irradiated at 10^5 ergs/mm^2 and hnRNP was isolated as described (15). RNA was purified from control and crosslinked particles by proteinase K digestion followed by phenol/chloroform extraction, and then it was electrophoresed in denaturing polyacrylamide gels. After electrophoresis, the RNA was transferred to diazobenzyloxymethyl-paper and hybridized with a ^{32}P-labeled DNA probe for β-globin sequences (see legend to Fig. 4 for details). It can be seen in Fig. 4 that the β-globin sequences from crosslinked Friend cell hnRNP (lane B) show no sign of degradation when compared

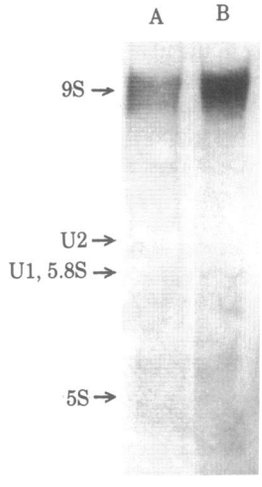

FIG. 4. Blot hybridization of β-globin RNA sequences in control and crosslinked hnRNP particles from Friend erythroleukemia cells. Unlabeled RNA purified from hnRNP was electrophoresed in a 7.5% polyacrylamide gel containing 98% (vol/vol) formamide. After electrophoresis, the RNA was electrophoretically transferred onto diazobenzyloxymethyl-paper (25) and hybridized with a ^{32}P-labeled nick-translated 1.05-kilobase *Hin*dIII restriction endonuclease fragment (15) of a mouse β-globin$_{major}$ gene cloned in bacteriophage λ (26). Lane A, RNA from noncrosslinked hnRNP. Lane B, RNA from hnRNP particles crosslinked in intact Friend cells. The reactive band corresponds to 9S β-globin mRNA sequences. Prespliced 15S β-globin sequences are also present in hnRNP (15) but were not resolved as a discrete band in this gel. The position of 9S β-globin RNA was determined by electrophoresis of Friend cell cytoplasmic poly(A)$^+$ RNA in a parallel lane, followed by blotting and hybridization with the β-globin DNA probe. The positions of smaller RNAs were determined by electrophoresis of ^{32}P-labeled Friend cell small nuclear RNAs in parallel.

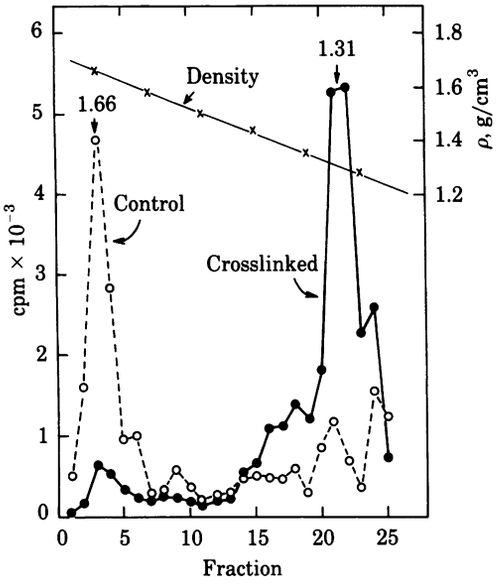

FIG. 5. Extent of hnRNA–protein crosslinking as measured by isopycnic banding in Cs_2SO_4. Samples (0.5 ml) of hnRNP crosslinked *in vivo* or hnRNP not crosslinked were mixed to homogeneity with 4.5 ml of Cs_2SO_4 having an initial density of 1.50 g/cm^3. The solutions were then pipetted into polyallomer tubes and centrifuged in a Beckman Spinco SW 50.1 rotor at 35,000 rpm for 72 hr (20°C). The gradients were fractionated, and the density of every fourth fraction was determined by weighing a 10-μl aliquot. [^{3}H]Uridine radioactivity was determined by precipitation with 5% trichloroacetic acid.

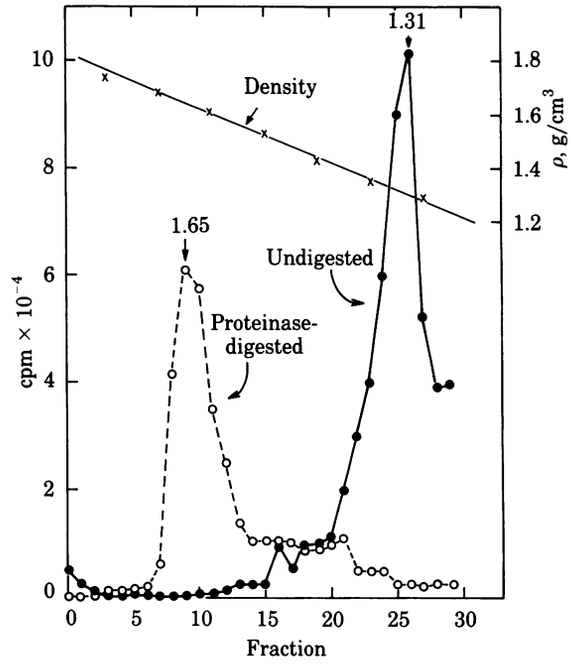

FIG. 6. Effect of proteolytic digestion on crosslinked hnRNP. Crosslinked particles in RSB were digested with proteinase K (200 μg/ml, 30 min, 37°C) and then layered on a preformed Cs_2SO_4 gradient (1.25–1.75 g/cm^3) and centrifuged in a SW 50.1 rotor at 34,000 rpm for 63.5 hr (20°C).

to RNA from noncrosslinked particles (lane A). This result not only demonstrates that the irradiation used for crosslinking does not significantly break hnRNA but also attests to the DNA hybridization competence of RNA from crosslinked hnRNP, which may prove to be an important attribute with respect to future studies on specific gene transcripts in hnRNP and their associated proteins (see *Discussion*).

Composition of Crosslinked hnRNP Particles as Measured by Isopycnic Banding in Cs_2SO_4. To investigate the RNA and protein composition of crosslinked hnRNP particles, they were banded in Cs_2SO_4 density gradients under conditions that dissociate noncrosslinked hnRNP. As shown in Fig. 5, when noncrosslinked hnRNP particles are mixed to homogeneity with Cs_2SO_4 (ρ = 1.50 g/cm^3) and then banded in a centrifuge-generated gradient, most of the hnRNA is stripped of protein and bands at 1.66 g/cm^3, which is the density of protein-free RNA in these gradients (27). In striking contrast, crosslinked hnRNP particles are completely resistant to this assault and band at a characteristic hnRNP density of 1.31 g/cm^3. To examine the remote possibility that irradiation *per se* chemically alters RNA to such an extent that it no longer has a density of 1.66 g/cm^3, naked hnRNA was irradiated at 3.6 × 10^5 ergs/mm^2 and then centrifuged in a Cs_2SO_4 gradient. This revealed that irradiated hnRNA retains its characteristic buoyant density of 1.66 g/cm^3 (data not shown). That the difference in buoyant density between crosslinked and noncrosslinked hnRNP in Fig. 5 is indeed due to hnRNA-associated protein is shown by the effect of protease treatment on crosslinked hnRNP, which converts the particles to naked RNA (Fig. 6).

We have previously shown that noncrosslinked hnRNP particles band at 1.32–1.35 g/cm^3 when layered on a *preformed* Cs_2SO_4 gradient (15, 28). In contrast to the situation in which particles are mixed into 1.50-g/cm^3 Cs_2SO_4 (Fig. 5), in preformed gradients the particles never reach a Cs_2SO_4 concentration high enough to promote their dissociation, because they first reach, and band at, their isopycnic density of 1.32–1.35 g/cm^3. Although it is possible that some protein is stripped from the particles as they reach 1.32 g/cm^3, the fact that the same density is observed when glutaraldehyde-fixed particles are banded in preformed gradients (23) leads us to take a density of 1.32–1.35 g/cm^3 as a reliable indicator of the actual protein-to-RNA mass ratio of hnRNP. This is very close to the density observed (1.31 g/cm^3) when crosslinked particles are banded under conditions that completely strip noncrosslinked hnRNP (Fig. 5). It therefore follows that essentially the same mass of protein that is usually isolated as "hnRNP" is also present in hnRNP particles that have been photochemically crosslinked *in vivo*.

DISCUSSION

The central objective of this study was to assess the biological authenticity of the isolated nuclear hnRNA·RNP complexes known as hnRNP particles (1). The results demonstrate that these particles represent native structures, because they can be shown to preexist in the unfractionated cell by photochemically catalyzed hnRNA–protein crosslinking *in vivo*. We cannot conclusively eliminate the possibility that 254-nm irradiation simply shifts a dynamic equilibrium of weak hnRNA–protein interactions in the direction of hnRNP rather than covalently stabilizing preexisting particles. However, because hnRNP particles can be observed to exist in the steady state by electron microscopy (4–10), we are inclined to believe that these structures are based upon high-affinity RNA–protein interactions.

The dose–response relationships of hnRNP crosslinking indicate a progressive increase in the fraction of hnRNA molecules that retain associated protein in the presence of 8 M urea and 0.5% sodium dodecyl sulfate (Fig. 1). The fact that the crosslinking curves span 3–4 orders of magnitude of UV dose raises the possibility that there are multiple classes of hnRNP having somewhat different structures, as was previously suggested on the basis of the effects of salt on their dissociation (12, 29). The heterogeneity in the dose–response curves may also reflect dif-

ferences in the specific UV sensitivities of various nucleotide–amino acid neighbors (16), which may vary in their prevalence among different classes of hnRNP. Finally, it is clear in Fig. 1 that higher doses are required to crosslink hnRNP in intact cells than in isolated nuclei or isolated particles. This is presumably due to absorption of the incident UV light by cellular components such as ribosomes, chromatin, and nucleotide pools. However, the dose used for crosslinking *in vivo* (3.6×10^5 ergs/mm^2) does not induce structural alterations in hnRNP by the criteria of nuclear fractionation behavior and recovery, sedimentation properties, pancreatic RNase digestion profiles (Fig. 2), the activity of a particle-bound enzyme system that may be involved in hnRNA → mRNA processing (Fig. 3), the covalent integrity of a specific mRNA sequence (Fig. 4), and the particles' RNA-to-protein ratio as determined by Cs_2SO_4 banding (Fig. 5). In addition, electrophoretic analyses of proteins from crosslinked hnRNP (30) reveal that the same set of hnRNP protein species previously identified (12) remains bound to the hnRNA in the presence of 0.5 M NaCl and 0.5% sodium dodecyl sulfate.

hnRNP crosslinking may be a useful vehicle for analyzing aspects of its structure or function that require the use of conditions that would normally disrupt particle integrity. For example, it may be possible to hybridize crosslinked hnRNP particles to filter-immobilized cloned DNA, because the crosslinked particles are likely to withstand the high salt employed for hybridization. Photochemical crosslinking also provides a means of stabilizing RNA–protein interactions during experiments in which protein mobility in the particles would complicate the analysis, for example in locating proteins on specific hnRNA sequences by nuclease protection (31).

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