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Heterogeneous nuclear RNA double-stranded regions probed in living HeLa cells by crosslinking with the psoralen derivative aminomethyltrioxsalen*

(secondary structure of inverted repeat heterogeneous nuclear RNA sequences/4′-aminomethyl-4,5′,8-trimethylpsoralen/covalent interstrand crosslinks/accessible RNA sites in nuclear ribonucleoprotein particles)

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ABSTRACT **The psoralen derivative aminomethyltrioxsalen (AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen) has been employed as a probe for heterogeneous nuclear RNA (hnRNA) double-stranded regions in experiments with living HeLa cells. hnRNA-ribonucleoprotein (hnRNP) particles were purified from untreated or AMT-treated cells after irradiation with 365-nm light, and double-stranded hnRNA regions (dsRNA) were isolated by RNase A+T1 digestion of hnRNP, followed by preparative Cs_2SO_4 isopycnic centrifugation. The purified, hnRNP-derived dsRNA was then assayed for interstrand crosslinks by measurement of its "snapback" to RNase-resistant form after thermal denaturation. By this procedure, the amount of crosslinked dsRNA was found to be increased 3- to 7-fold in cells exposed to AMT *in vivo*. The levels of crosslinking *in vivo* compared favorably with those observed in model experiments with pure dsRNA *in vitro*. These results establish that double-stranded hnRNA regions exist in the living cell, and they further demonstrate that these base-paired regions are organized as rather accessible sites within the nucleus.**

Eukaryotic primary gene transcripts and their metabolic descendents, collectively known as heterogeneous nuclear RNA (hnRNA), undergo a number of post-transcriptional processing modifications before their emergence from the nucleus as finished messenger RNA (mRNA) (reviewed in ref. 1). Regions of secondary structure in hnRNA might function in some of these modifications, by serving as recognition sites for processing enzymes (2–4). Stable, RNase-resistant double-stranded RNA (dsRNA) regions have been isolated from the hnRNA of a variety of cell types and organisms (5–7) but, in contrast, cytoplasmic mRNA contains almost no double-stranded material (7, 8). However, the existence of sequence homology between regions of mRNA and dsRNA isolated from hnRNA (9–12) suggests that the double stranded regions of hnRNA may undergo enzymatic cleavages that conserve a portion of their primary nucleotide sequence in mRNA, a situation consistent with their proposed role in processing.

Another aspect of hnRNA that must be considered in any contemporary treatment of its processing is the fact that hnRNA possesses a highly ordered ribonucleoprotein structure *in vivo* (13, 14). We have been investigating this ribonucleoprotein organization in the belief that an important key to understanding hnRNA processing lies in its specific interactions with nuclear proteins. We have proposed that one important function of hnRNA-bound proteins might be to modulate the formation of double-stranded regions for processing cleavages (3, 4). We view hnRNA-bound proteins as restricting the formation of double-stranded regions between some, but not all, of the potentially base-paired sequences in hnRNA.

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As a first step toward exploring this model, we have made use of the psoralen derivative aminomethyltrioxsalen (AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen) as a probe for the existence and accessibility of hnRNA secondary structure in the intact HeLa cell. The psoralens are bifunctional nucleic acid intercalating agents that can penetrate intact cells and form interstrand crosslinks between pyrimidine residues of nucleic acid helices upon irradiation with ultraviolet light (365 nm). Psoralen derivatives have been used to map and determine the secondary structure of inverted repeat sequences in cellular and viral DNA (15–17) and in ribosomal RNA *in vitro* (18). A particularly attractive feature of the psoralens, revealed by their use as probes of chromatin structure (19–22), is their ability to react with regions of nucleic acid secondary structure *in vivo*, without apparently disturbing native nucleoprotein organization. In the present study we report that psoralens can be used to crosslink double-stranded regions of hnRNA in intact cells, showing that hnRNA secondary structure exists in the living cell.

EXPERIMENTAL PROCEDURES

Synthesis of 4′-Aminomethyl-4,5′,8-trimethylpsoralen. 4,5′,8-Trimethylpsoralen (1.0 g, a gift of the Paul Elder Co., Bryan, OH) was sequentially converted to 4′-chloromethyl-4,5′,8-trimethylpsoralen and 4′-*N*-phthalimidomethyl-4,5′,-8-trimethylpsoralen as detailed (23). 4′-Aminomethyl-4,5′,-8-trimethylpsoralen was then obtained from the phthalimidomethyl product in 19% yield by reaction with hydrazine: mp 258–270°C; NMR (C^2HCl_3) as the free amine chemical shifts δ in ppm relative to tetramethylsilane 1.38–1.57 (2 H, broad singlet, $-CH_2NH_2$), 2.50–2.55 (9 H, multiplet, C4, 5′, and 8 methyls), 3.96 (2 H, singlet, $-CH_2NH_2$), 6.27 (1 H, singlet, C3 H), 7.57 (1 H, singlet, C5 H). This synthesis was carried out in collaboration with Marcel Gut of this institute. The compound was dissolved in buffered isotonic saline (0.15 M NaCl/0.01 M Tris·HCl, pH 7.2) at a concentration of 2.0 mg/ml or dilutions thereof, depending upon the experiment.

Crosslinking Protocol. HeLa cells (S_3 strain) grown as described (24) were pulse-labeled for 20 min at 37°C with [^{3}H]-uridine [25 μCi/ml (1 Ci = 3.7×10^{10} becquerels)] in the presence of 0.04 μg of actinomycin per ml (14). Labeling was quenched by rapidly washing the cells twice in Earle's balanced salt solution (without phenol red) at room temperature (22–

Abbreviations: hnRNA, heterogeneous nuclear RNA; dsRNA, double-stranded RNA; hnRNP, hnRNA·ribonucleoprotein; AMT, aminomethyltrioxsalen (4′-aminomethyl-4,5′,8-trimethylpsoralen).

* This is paper 10 in a series entitled "Ribonucleoprotein organization of eukaryotic RNA." Paper 8 is ref. 4, and paper 9 is submitted for publication. An account of this work has been published in abstract form (41).

25°C), followed by a third wash in an isotonic buffer (0.15 M NaCl/0.01 M Tris·HCl, pH 7.2). The cells were then divided into three equal-sized portions for AMT treatment. Each pellet, which contained approximately 3×10^8 cells, was resuspended in 2 ml of buffered saline containing either no AMT (control), 0.2 mg of AMT per ml, or 2.0 mg of AMT per ml, and the cell suspensions were then incubated at 22–25°C for 10 min. For irradiation, the cell suspensions were transferred to 30-ml Falcon tissue culture flasks (no. 3012) and rapidly chilled to 4°C. The flasks were placed horizontally 5 mm over the filter surface of a long-wave ultraviolet illuminator (Chromato-Vue Transilluminator, Model C-62, Ultraviolet Products, San Gabriel, CA) and irradiated with light having a peak emission of 365 nm and an energy flux of 6000 $\mu W/cm^2$ at the filter surface. Irradiation was carried out for 1.5 hr in the cold room (4°C), and the flasks were kept chilled during the irradiation by using a fan to constantly direct cold air over and under the flasks. Cell fractionation and hnRNP isolation were carried out according to our published methods (14, 25).

Preparation of dsRNA. Sucrose gradient-purified hnRNA·ribonucleoprotein (hnRNP) particles (14) were dialyzed against 0.01 M NaCl/1.5 mM $MgCl_2$/0.01 M Tris·HCl, pH 7.0, and then made 0.5 M in NaCl and 0.1 mM in $CaCl_2$. Pancreatic RNase (10 μg/ml), RNase T1 (0.1 μg/ml), and iodoacetamide-treated (26) pancreatic DNase (5 μg/ml) were added, and the hnRNP was incubated for 1.5 hr at 37°C. Sodium dodecyl sulfate (0.5%) and proteinase K (100 μg/ml) were added and the incubation was continued at 22–25°C for 2 hr. The limit digest, consisting of double-stranded RNA (4), was then extracted twice with phenol/chloroform/isoamyl alcohol (50:49.5:0.5, vol/vol) and precipitated with ethanol.

Cs_2SO_4 Fractionation of dsRNA from AMT-Treated Cells. dsRNA from control cells (UV-irradiated) and cells treated with 0.2 mg of AMT per ml and 2.0 mg of AMT per ml was dissolved in 0.5 M NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.5, (TEN). For analytical Cs_2SO_4 gradients a small portion of each dsRNA sample was diluted to 2.5 ml with TEN and layered on 4 ml of Cs_2SO_4 having an initial density of 1.75 g/cm^3 in polycarbonate centrifuge tubes, and the mixture was gently swirled together. The remaining (2.5 ml) undiluted portion of each sample was treated in a similar manner for preparative gradients centrifuged in parallel. Centrifugation was for 82 hr at 44,000 rpm (20°C) in the Spinco 50 Ti angle rotor. Fractions from the analytical gradients were precipitated with trichloroacetic acid and the radioactivity on filters was measured to locate the peak of dsRNA. Fractions from the preparative gradients were pooled as indicated in Fig. 2 and were then dialyzed against 10 mM NaCl/10 mM Tris·HCl, pH 7.2.

RESULTS

Crosslinking of Isolated dsRNA *In Vitro*. As a model experiment to determine whether the double-stranded regions of hnRNA have the capacity to be crosslinked with AMT, dsRNA was isolated from hnRNP particles by RNase digestion and phenol deproteinization (4) and subjected to the crosslinked protocol, as a purified nucleic acid *in vitro*. We defined dsRNA by the combined criteria of sensitivity to RNase at low ionic strength and resistance to RNase before denaturation, and we scored as snapback the fraction of this radioactive dsRNA that regained RNase resistance after denaturation and quick cooling. In principle, a single AMT crosslink at any point along an RNA double helix should permit the duplex to snap back. As shown in Table 1, the non-irradiated and UV-irradiated controls were found to have snapback fractions of 9.8% and 9.1%, respectively. (This material is probably a small population of dsRNA molecules that have intact single-strand connecting loops. If sufficiently short, these connecting segments could survive the RNase digestions involved in the isolation of dsRNA from hnRNP. It is also conceivable that some or all of the apparent snapback fraction of the untreated dsRNA actually represents second-order reassociation between very abundant, low complexity dsRNA sequences, rather than first-order renaturation.) In contrast to the controls, the AMT-treated dsRNA was found to have a much larger snapback fraction, 39.6%, which is approximately 4.4-fold larger than the UV-irradiated control (Table 1). This shows that a substantial number of hnRNP-derived dsRNA molecules have the capacity to be crosslinked *in vitro* by AMT. The fact that the observed level of crosslinking is less than 100% may be due to the particular reaction conditions employed. However, the results shown in Table 1 point to the feasibility of attempting to crosslink double-stranded hnRNA sequences *in vivo*, and moreover, provide a useful point of reference with which we can compare the *in vivo* results.

Table 1. *In vitro* crosslinking of dsRNA

Treatment AMT	Treatment UV	RNase-resistant RNA, cpm A High-salt, native	B High-salt, snapback	C Low-salt	D $\frac{B-C}{A-C}$, %	E Fold increase
–	–	880	117	34	9.8	
–	+	1016	121	31	9.1	
+	+	968	395	20	39.6	4.4

dsRNA isolated from hnRNP particles was deproteinized, dissolved in 0.15 M NaCl/0.01 M Tris·HCl, pH 7.2, and divided into three 1-ml samples for an unirradiated control, a UV-irradiated control, and for treatment with 50 μg of AMT per ml plus UV irradiation. After treatment the samples were ethanol precipitated twice and dissolved in 10 mM NaCl/10 mM Tris·HCl, pH 7.2. High-salt samples were then made 0.5 M in NaCl. Duplicate 1-ml volumes of the samples to be assayed for snapback (column B) and RNase resistance at low ionic strength (column C) were denatured at 100°C for 5 min and rapidly cooled to 0°C in an ethanol/dry ice bath. Nondenatured samples (column A) were also cooled to 0°C. All samples were then digested with 10 μg of RNase A per ml and 0.1 μg of RNase T1 per ml for 1.5 hr at 37°C, precipitated with trichloroacetic acid, and assayed on filters for radioactivity. Values of RNase-resistant cpm are the averages of duplicates after background has been subtracted. Snapback dsRNA (column D) is determined by the combined criteria of RNase resistance (after heat denaturation and quick cooling) at high ionic strength (column B), and RNase sensitivity at low ionic strength (column C) relative to the RNase-resistant dsRNA radioactivity at high ionic strength of a nondenatured sample (column A minus column C). Fold increase (column E) was calculated for the AMT-treated sample relative to the UV-irradiated control.

Isolation of hnRNP and dsRNA from AMT-Treated Cells. We next asked whether it is possible to isolate hnRNP particles, as well as hnRNP-derived dsRNA, from cells that had been subjected to AMT treatment and UV irradiation. Table 2 shows the results of a representative experiment in which a culture of HeLa cells was pulse labeled with [^{3}H]uridine and then divided into several portions for the crosslinking protocol. Results are shown for untreated cells, cells that were UV irradiated only, and cells exposed to two different concentrations of AMT. It can be seen that the recovery of hnRNP particles, defined as [^{3}H]uridine radioactivity, was similar for all four samples. This indicates that neither AMT treatment nor UV irradiation has gross effects on subcellular organization, at least not as manifested by one's ability to perform cell or nuclear fractionation. In addition, the amounts of RNase-resistant dsRNA obtained from all four preparations were similar to those we have previously reported (3, 4). Moreover, the deproteinized dsRNA isolated from AMT-treated cells displayed a Cs_2SO_4 buoyant density typical of double-stranded RNA, as will be shown in the

Table 2. Recovery of hnRNP and hnRNP-derived dsRNA from AMT-treated cells

Treatment		hnRNP recovery, cpm per 2.8×10^8 cells	dsRNA recovery, % RNase A+T1-resistant
AMT	UV		
–	–	3,964,000	1.9
–	+	6,913,350	1.5
0.2 mg/ml	+	5,740,200	1.8
2.0 mg/ml	+	6,232,950	3.6

hnRNP was isolated from HeLa cells that were untreated, UV irradiated only, or treated with AMT at 0.2 or 2.0 mg/ml and then UV irradiated. hnRNP recovery was determined by measuring radioactivity in an aliquot of sucrose gradient-purified hnRNP particles. The hnRNP was then digested with RNase at high ionic strength. dsRNA recovery is expressed as trichloroacetic acid-insoluble radioactivity in the RNase-digested fraction relative to the undigested hnRNP.

next section. Thus, the hnRNP particles and the hnRNP-derived dsRNA isolated after AMT crosslinking *in vivo* appear to be normal in all respects examined.

Purification of dsRNA from hnRNP by Preparative Cs_2SO_4 Gradient Centrifugation. The assay for crosslinking described above measures the RNase resistance due to snapback against a small background of radioactivity that is resistant to RNase at low ionic strength. Therefore the assay deals effectively and quantitatively with any contaminating DNA ("mislabeled" by [^{3}H]uridine via [^{3}H]deoxycytidylate), or with single-stranded RNA that has reacted with AMT to an extent that it is resistant to RNase even at low ionic strength, a situation that we have shown can indeed occur when isolated hnRNP is treated with very high levels of AMT and irradiated *in vitro* (data not shown). However, the assay cannot discriminate between RNA duplexes and DNA·RNA hybrids. While we have no direct evidence that the latter are present in our preparations, it was, at least at the outset, a distinct possibility. It is known that AMT can crosslink DNA·RNA hybrids *in vitro*, including those that arise between nascent RNA and the DNA template during transcription (27, 28). Although the isolated hnRNP particles being employed here are free of chromatin (14, 25), it is possible that DNA·nascent hnRNA hybrids are sheared from the chromatin during the isolation of hnRNP. The RNA strands of these putative hybrids would be RNase resistant at high ionic strength, RNase-sensitive at low ionic strength, and, if crosslinked to their complementary DNA chains by AMT, would be scored as such in the snapback assay. Although this possibility is rendered rather unlikely by the deliberate inclusion of a pancreatic DNase digestion step in the standard dsRNA isolation procedure, we nevertheless elected to purify the dsRNA further by preparative Cs_2SO_4 density gradient centrifugation under conditions that resolve RNA duplexes from DNA·RNA hybrids. Fig. 1 shows Cs_2SO_4 gradient profiles for hnRNP-derived dsRNA and for DNA·RNA hybrids. The latter were constructed by hybridizing a ^{3}H-labeled cDNA, reverse transcribed from HeLa poly(A)-containing mRNA, with a vast excess of hnRNA, and then digesting single-stranded RNA and cDNA tails with S1 nuclease. Therefore, these hybrids can be considered as being composed of an equal mass of RNA and DNA. The hybrids banded at 1.48 g/cm^3, while the hnRNP-derived dsRNA banded at 1.57 g/cm^3, which was also the density of pure dsRNA standards run in parallel gradients. It can be seen that the DNA·RNA hybrids are sufficiently resolved from the dsRNA region of the gradient so that with an appropriate fractionation protocol they can be almost completely eliminated (Fig. 1). If, for example, fractions 3–11 in Fig. 1 are pooled for dsRNA, and fractions 12–20 are taken to represent DNA·RNA hybrids, then it can be readily computed that not

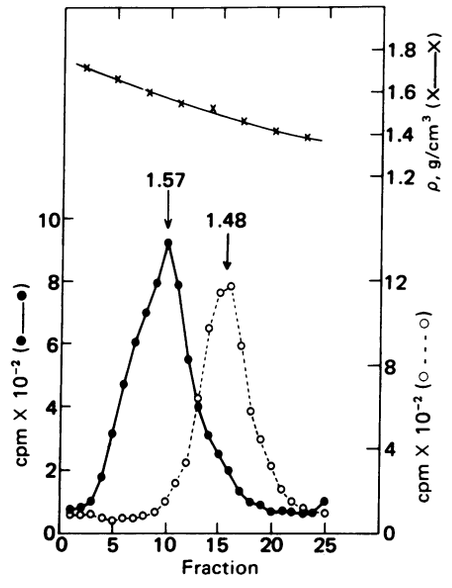

FIG. 1. Resolution of hnRNP-derived dsRNA from DNA·RNA hybrids by equilibrium Cs_2SO_4 density gradient centrifugation. [^{3}H]Uridine-labeled dsRNA was isolated from hnRNP particles by limit RNase A+T1 digestion and phenol deproteinization. DNA·RNA hybrids were constructed by annealing an excess of HeLa cell hnRNA with a trace of [^{3}H]cDNA reverse transcribed from HeLa poly(A)-containing mRNA and then digesting single-stranded cDNA and RNA with S1 nuclease. The samples were centrifuged on separate Cs_2SO_4 gradients and are plotted together in the figure to facilitate comparison. Centrifugation was in the Spinco 50 Ti fixed-angle rotor at 44,000 rpm for 90 hr (20°C). ●, dsRNA; ○, DNA·RNA hybrid; X, density.

more than about 6% of the radioactivity present in any putative hybrids will be recovered in the pooled dsRNA fractions. (We again emphasize that we have no evidence that hybrids are present in the experimental material itself.)

Crosslinking of hnRNA Double-Stranded Regions *In Vivo*. hnRNP particles were isolated from control and AMT-treated cells and digested with RNases A and T1 in the presence of pancreatic DNase. The digests were deproteinized and then centrifuged on preparative Cs_2SO_4 gradients (Fig. 2 *A–C*). Fractions 3–11 of the preparative gradients, as delineated by the vertical lines under the analytical gradient profiles in *A–C*, were pooled and used to determine the extent to which AMT treatment had crosslinked double-stranded hnRNA sequences *in vivo*. For comparison, fractions 12–20 of these gradients (which might contain DNA·RNA hybrids, see Fig. 1), were also pooled and assayed. The results of the snapback measurements are shown in Table 3. Considering first the material banding as pure dsRNA (ρ = 1.57–1.58 g/cm^3, fractions 3–11), it can be seen that, in untreated (but UV-irradiated) HeLa cells, this dsRNA had a snapback fraction of 2.6%. However, the amount of crosslinked dsRNA observed in cells treated with AMT at 0.2 or 2.0 mg/ml was markedly increased to 8.6% of the total dsRNA with AMT at 0.2 mg/ml, and to 18.7% of the total dsRNA with AMT at 2.0 mg/ml, representing increases over the control of 3.3- and 7.2-fold, respectively. This is also illustrated by the histogram in Fig. 2*D*, which shows the absolute amounts of RNase-resistant radioactive material measured in both low and high salt for the pooled gradient fractions of *A–C*. The results specifically for the ρ = 1.57–1.58 g/cm^3 dsRNA fractions of *A*, *B*, and *C* are represented by the histogram bars directly below their corresponding gradient regions, and are numbered pools 1, 3, and 5, respectively. The heights of these bars illustrate the progressive increase in the absolute levels of

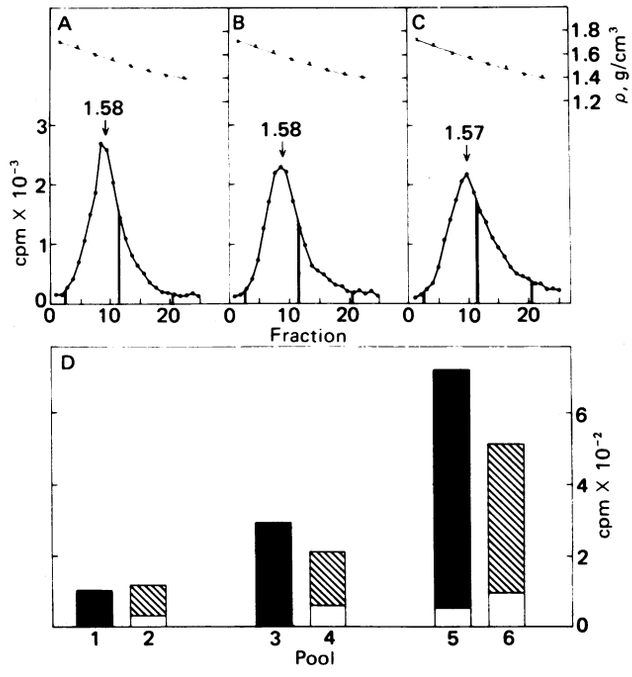

FIG. 2. Preparative Cs_2SO_4 centrifugation of dsRNA from control and AMT-treated cells and assay of crosslinking. hnRNP-derived dsRNA from control cells (*A*) and from cells treated with AMT at 0.2 mg/ml (*B*) and 2.0 mg/ml (*C*) was centrifuged on parallel sets of analytical and preparative Cs_2SO_4 gradients. Fractions 3–11 and 12–20 from the preparative gradients were pooled as delineated by the vertical lines under the profiles in *A–C*. The pooled gradient fractions were then analyzed for the presence of crosslinked RNA molecules as described for Table 1. The total amount of [^{3}H]uridine radioactivity in the snapback fraction of each gradient region is illustrated by the heights of the vertical bars in *D*. The shaded portions (solid or hatched) of each bar represent the amount of snapback dsRNA as defined by the combined criteria of RNase resistance at high ionic strength and RNase sensitivity in low ionic strength (see *Results* for additional details). An increase in the shaded portion of each bar relative to controls (pools 3 and 5 compared with pool 1, and pools 4 and 6 compared with pool 2) is taken as a measure of AMT crosslinking (see also Table 3).

crosslinked dsRNA observed in cells treated with increasing concentrations of AMT *in vivo*.

As shown in Table 3, the material banding on the lower density side of the main dsRNA peak (fractions 12–20) also reacted to a significant extent with AMT. In the absence of AMT treatment, this dsRNA had a snapback value of 12.6%, and this was increased to 20.6% and 42.6% by AMT at 0.2 and 2.0 mg/ml, respectively. The absolute amounts of AMT crosslinked (i.e., snapback) dsRNA banding at 1.48 g/cm^3 were considerably less than the amount observed in the 1.57–1.58 g/cm^3 material (compare height of bar 3 with bar 4 and bar 5 with bar 6 in Fig. 2*D*). From this it follows that the crosslinking due to AMT observed in fractions 3–11 cannot be due to spillover into this density region of some non-dsRNA component (such as the heavier edge of an isopycnic band of putative DNA·RNA hybrids at 1.48 g/cm^3—see Fig. 1).

In summary, we conclude from these data (Fig. 2 and Table 3) that base-paired, RNase-resistant regions exist in hnRNA in the living cell and, moreover, that they are organized within the ribonucleoprotein structure of hnRNA in a highly accessible configuration *in situ*.

DISCUSSION

hnRNA Secondary Structure in Relation to mRNA Processing. The concept of RNA processing finds its roots in the discovery of large precursor molecules for eukaryotic ribosomal RNA (29, 30). The existence of RNA processing from larger precursors has been established for a number of prokaryotic, viral, and eukaryotic RNAs (reviewed in ref. 1). Regions of secondary structure are known to be sites for endonucleolytic cleavages in precursors to *Escherichia coli* tRNA and rRNA as well as in maturation of bacteriophage T7 early mRNA, and a dsRNA-selective nuclease, RNase III, has been implicated in these events (31, 32). In the case of eukaryotes, the fact that mRNA can be shown to possess sequence homology with hnRNA-derived dsRNA (9–12) constitutes provocative evidence (but not proof) for an involvement of the double-stranded regions of hnRNA in mRNA processing. One of several possibilities is that hnRNA secondary structure might be involved in the post-transcriptional juxtaposition of coding regions in transcripts of structural genes that contain intervening sequences. Regions of potential RNA secondary structure can be deduced from the DNA sequences flanking known intron–exon junctions in the genes coding for a mouse λ_{II} immunoglobulin light chain (33) and chicken ovalbumin (34), although these hairpins would contain some base mismatching. In this context, it is of interest to note that we previously demonstrated (3) that there are two classes of double-stranded RNA in HeLa hnRNP particles probed with the dsRNA-selective nuclease *E. coli* RNase III. Both classes of double-stranded RNA were shown

Table 3. *In vivo* crosslinking of dsRNA

		RNase-resistant RNA, cpm				
		A	B		D	E
Cs_2SO_4 fractions	AMT	High-salt, native	High-salt, snapback	C Low-salt	$\frac{B-C}{A-C}$, %	Fold increase
3–11	None	3737	103	6	2.6	
	0.2 mg/ml	3397	293	2	8.6	3.3
	2.0 mg/ml	3616	719	51	18.7	7.2
12–20	None	720	118	31	12.6	
	0.2 mg/ml	790	211	61	20.6	1.6
	2.0 mg/ml	1080	514	94	42.6	3.4

dsRNA isolated from the hnRNP of control (UV-irradiated) cells and cells treated with AMT at 0.2 or 2.0 mg/ml was fractionated on preparative Cs_2SO_4 gradients as shown in Fig. 2. Pooled gradient fractions 3–11, corresponding to the dsRNA peak (pools 1, 3, and 5 in Fig. 2), and fractions 12–20 (pools 2, 4, and 6 in Fig. 2) were dialyzed against 10 mM NaCl/10 mM Tris·HCl, pH 7.2. Samples were then assayed for snapback dsRNA as detailed in Table 1. The results of the snapback assays are also shown in Fig. 2*D*, in which the bar heights represent total radioactivity in each snapback fraction (column B in this table) and the shaded (solid or hatched) portion of each bar represents radioactivity in the snapback dsRNA fraction (column B minus column C).

to be RNase III-sensitive at moderate ionic strength, but one of these was found to be resistant to RNase A+T1 digestion only at high ionic strength, suggesting the presence of double-stranded regions of differing stability in the native hnRNP particle (3). Finally, the presence of dsRNA-selective nucleases in eukaryotes (35–37) further points to a role of double-stranded regions in RNA metabolism, although the particular species of cellular RNA upon which these enzymes act has not yet been determined..

Existence of hnRNA Secondary Structure *In Vivo*. Although there may be no reason to doubt *a priori* the intracellular existence of secondary structure in RNA species that exist in the cell as protein-free polynucleotides, the issue is far more critical in the case of RNAs that are known to possess an extensive ribonucleoprotein structure, such as hnRNA. Thus, one could not have assumed that the double-stranded sequences described in deproteinized extracts of hnRNA (5–7) necessarily exist *in vivo*. Moreover, the existence of hnRNA secondary structure *in vivo* takes on added importance in light of the fact that there are single-stranded self-complementary sequences in hnRNA (38–40) that are apparently constrained in a single-stranded form by their association with proteins in the hnRNP particles (discussed in ref. 3). This serves to further underscore the importance of critically determining whether or not there is any dsRNA at all in hnRNP *in vivo*. To this end we have used the psoralen derivative AMT to demonstrate the existence of hnRNA secondary structure in intact HeLa cells.

Another, related issue addressed by these studies is the accessibility of double-stranded hnRNA sequences to the AMT probe *in vivo*. While we knew that the double-stranded regions of *isolated* hnRNP particles are largely free of bound protein (4), it was entirely possible that these regions are much more extensively complexed with protein in the cell. Our results show that this is not the case for at least a substantial fraction of the double-stranded regions of hnRNP *in vivo*, because these are capable of being intercalated and crosslinked by AMT. We obviously cannot at this point make any conclusions for the fraction of hnRNP-derived dsRNA that is not crosslinked by AMT in these experiments. There are, of course, limits on the intracellular concentrations of AMT that can be achieved *in vivo*, and in fact the highest concentration we have employed here (2.0 mg/ml) is one approaching the aqueous solubility limit of this compound (23). Considering the plasma membrane and nuclear envelope permeability barriers, and the rheological and metabolic complexities of the intact cell, the *in vivo* levels of AMT crosslinking observed compare favorably with the level obtained with pure dsRNA *in vitro* (Table 1). Further insights should be provided by comparative studies of crosslinking of hnRNP *in vivo* and *in vitro*, combined with analysis of AMT binding levels by using radioactively labeled AMT, prompted by the present demonstration that hnRNA double-stranded regions do indeed exist *in vivo*.

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