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Double-Stranded Regions in Heterogeneous Nuclear RNA from Hela Cells

(messenger RNA/intramolecular base pairing/RNase resistance)

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ABSTRACT **Heterogeneous nuclear RNA from HeLa cells contains double-stranded regions that arise by base pairing of complementary sequences that exist as parts of the same molecule (intramolecular base pairing). When denatured, the RNA sequences that form the double-stranded regions hybridize rapidly to HeLa cell DNA, suggesting that they are transcribed from reiterated sites in the genome. The messenger RNA does not contain the same class or amount of double-stranded RNA regions found in heterogeneous nuclear RNA.**

Mammalian cells contain a high molecular weight nuclear RNA that varies in size and has a "DNA-like" base composition (1–5). The relationship of this heterogeneous nuclear RNA (HnRNA) to the cytoplasmic messenger RNA (mRNA) has been intensively studied for several years. Although it seems clear that most of the HnRNA is degraded in the nucleus (1, 2, 6) and thus never reaches the cytoplasm, two recent findings suggest that a mechanism involving post-transcriptional modification of HnRNA may be involved in selecting some HnRNA sequences destined to become cytoplasmic mRNA. (*i*) High molecular weight HnRNA from DNA virus-transformed cells contains virus-specific sequences, while polyribosomal mRNA contains viral-specific sequences in lower molecular weight molecules (7, 8). (*ii*) Both the HnRNA and mRNA from HeLa cells contain a polyadenylic acid [poly(A)] sequence (9–11). Furthermore, inhibition of the addition of poly(A) to HnRNA blocks the appearance of most of the labeled mRNA in polyribosomes (12, 13).

Poly(A) sequences from large molecules can be studied because poly(A) resists endonuclease digestion under conditions where the major portion of the RNA molecule is destroyed (14). While studying the relationship between HnRNA and mRNA by analysis of poly(A) content, we encountered another type of sequence resistant to RNase, a double-stranded region, in HnRNA molecules. This double-stranded region is probably identical to that discovered by Montaignier (15) and studied by other workers (16–18). One important recent finding is that a portion of the double-stranded RNA isolated from total rat liver RNA hybridizes to homologous cellular DNA (16).

We report here that double-stranded RNA sequences are intramolecular in HnRNA molecules from HeLa cells and are absent from mRNA. When the isolated double-stranded RNA resistant to RNase is denatured, it hybridizes very rapidly to DNA, similar to rapidly hybridizing HnRNA sequences (27, 28). It seems likely that a study of the distribution within the HnRNA of these double-stranded RNA regions will prove useful in understanding the nature of HnRNA.

Abbreviations: HnRNA, heterogeneous nuclear RNA; Me_2SO, dimethylsulfoxide.

METHODS AND MATERIALS

The growth and labeling of HeLa cells has been described, as has the preparation of polyribosomes and nuclei and the extraction of RNA from cell fractions (19, 20). Analysis of base composition was done on alkaline hydrolysates of RNA as described by Sebring and Salzman (21). Cs_2SO_4 equilibrium density centrifugation was performed in the SW65 rotor of the Spinco L2 65B ultracentrifuge for 72 hr at 40,000 rpm and 25°. Gel electrophoresis through 15 or 20% polyacrylamide gels was performed on RNA samples in Tris-borate buffer (22). Hydroxyapatite adsorption of RNA samples was performed in 50 mM PO_4 buffer (pH 7.0) and elution was achieved by 0.5 M PO_4 buffer (23). RNA was hybridized to DNA bound to nitrocellulose filters as described (24).

RESULTS

The existence of a non-poly(A) fraction resistant to RNase in HnRNA from HeLa cells and its characterization as double-stranded RNA will be considered first, followed by a description of experiments designed to elucidate the nature of the relationship between double-stranded RNA and HnRNA. 95–97% of radioactive HnRNA (labeled with [^{3}H]uridine, [^{3}H]adenosine, or ^{32}P) sedimenting faster than 32 S in sucrose gradients could be digested (30 min, 37°) by ribonuclease (2 μg/ml) to acid-soluble form in a solution containing 0.1 M NaCl–10 mM Tris·HCl (pH 7.4)–10 mM EDTA (11). As a control, labeled double-stranded poliovirus RNA was digested under the same conditions and remained completely acid-precipitable. In order to rid the cellular RNA digests of low molecular weight products, the ribonuclease-resistant fraction was adsorbed to hydroxyapatite in a low-salt concentration (50 mM $PO_4^{=}$, pH 7.0) and eluted from the column in high salt concentration (0.5 M $PO_4^{=}$, pH 7.0). This procedure should allow recovery of double-stranded nucleic acid (23). Almost all of the RNase-resistant, acid-precipitable material was recovered in this step, while acid-soluble material was removed. After removal of excess salt by passage through Sephadex G-25, the material bound to hydroxyapatite migrated in a rather broad zone during electrophoresis in 15% polyacrylamide gels (Fig. 1). After electrophoresis, the entire band of RNase-resistant material was eluted from the gel and characterized as double-stranded RNA on the basis of several kinds of determinations. (*i*) When subjected to Cs_2SO_4 equilibrium density sedimentation, the material had the same

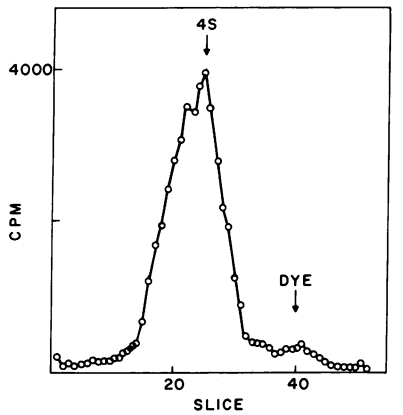

FIG. 1. [^{3}H]adenosine-labeled HnRNA was recovered from the 70–100S region of a sucrose gradient and digested with 2.5 μg/ml pancreatic and 5 units/ml of T1 ribonuclease in a buffer containing 0.1 M NaCl–10 mM Tris·HCl (pH 7.4)–10 mM EDTA at 37° for 30 min. The ribonuclease-resistant fraction was purified and subjected to electrophoresis in a 15% polyacrylamide gel along with ^{14}C-labeled 4S RNA from HeLa cells. After electrophoresis, the gel was fractionated into 2-mm slices for assay of radioactivity (22).

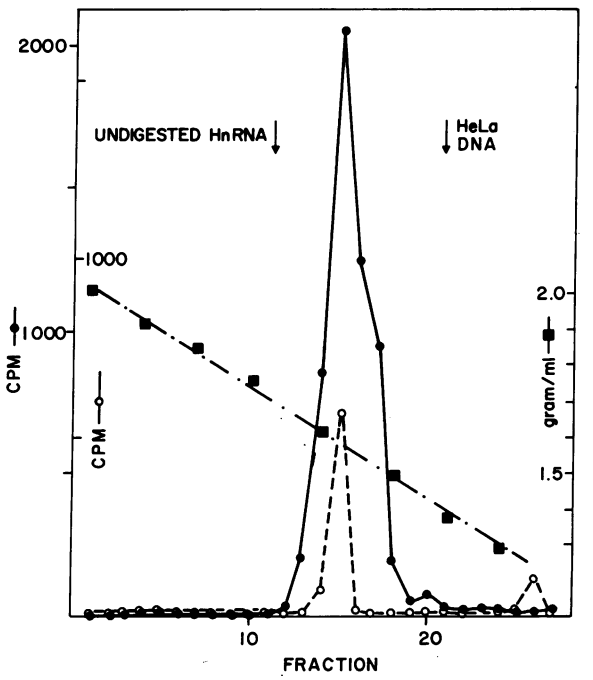

FIG. 2. ^{32}P-labeled, ribonuclease-resistant RNA prepared from HnRNA from HeLa cells eluted from a 15% acrylamide gel and (see Fig. 1) subjected to Cs_2SO_4 equilibrium density sedimentation along with [^{3}H]uridine-labeled reovirus RNA (kind gift of Dr. A. Shatkin). The RNA sample was layered on top of a preformed 4.5-ml linear gradient of Cs_2SO_4 ranging in density from 1.4–1.8 g/ml. Centrifugation was at 40,000 rpm in a Beckman SW65 rotor for 72 hr at 25°, after which 5-drop fractions were collected by tube puncture. The density of the fractions was determined by weighing a constant volume from each in a calibrated micropipette. Each fraction was then precipitated with trichloracetic acid and assayed for radioactivity. The *arrows* indicate the density at which HeLa cell DNA and undigested HnRNA banded in a parallel gradient. ●, reovirus RNA; O, RNase-resistant RNA from HnRNA from HeLa cells.

TABLE 1.

Exp.	HnRNA tested for double-stranded RNA	Total cpm	cpm Recovered from hydroxy-apatite	% Double-stranded
1	100S	1,296,000	46,100	3.2
	50 S	2,025,140	71,100	3.4
2*a*	Heated*	3,000,000	22,800	0.8
b	Heated†	3,000,000	86,400	2.9
3	Treated with 90% Me_2SO	4,000,000	135,000	3.4
4	LiCl precipitate	3,360,000	97,000	2.9
	LiCl supernatant	60,000	—	—
	HnRNA, after nuclease			
	LiCl precipitate	12,000	800	6.7
	LiCl supernatant	120,000	84,000	60

* 100° in 0.1 × SSC, then cooled to 0° in 2 × SSC for 1 min. SSC, 0.15 M NaCl–0.015 M Na citrate.

† 100° in 0.1 × SSC, cooled to 0°, and heated to 37° in 2 × SSC for 1 min.

buoyant density as reovirus RNA, a known double-stranded RNA (Fig. 2). (*ii*) ^{32}P-labeled RNase-resistant RNA, purified as described above, showed a "base-paired" base composition of 28.7% GMP, 28.6% CMP, 21.2% AMP, and 21.2% UMP (average of three determinations). (*iii*) That all the labeled material resistant to RNase in high-salt concentration was RNA was shown by its complete digestion by ribonuclease (30 min, 37°) at a low-salt concentration (15 mM NaCl–1.5 mM Na citrate) (20) and by its total conversion to ribonucleotides by KOH (alkali digestion for the base composition analysis). From these results we conclude that the ribonuclease-resistant fraction is double-stranded RNA. It may be assayed as acid-precipitable RNA by either adsorption and elution from hydroxyapatite or by gel electrophoresis after column elution. In most experiments reported in this paper we have used both assays.

Further experiments indicate that the double-stranded RNA was part of HnRNA molecules. Samples of [^{3}H]uridine-labeled HnRNA larger than 45 S were precipitated with 2 M lithium chloride either before or after digestion with ribonuclease, and double-stranded RNA was assayed in the supernatant and precipitate fractions (Table 1, Exp. 4). Before digestion with ribonuclease, *all* of the double-stranded material was found in the lithium chloride precipitate; after digestion *all* of the double-stranded material was found in the lithium chloride supernatant, indicating that the double-stranded regions exist as part of large, predominantly single-stranded molecules. Kimble and Duesberg (18) have reported that double-stranded RNA is found in the LiCl precipitate when total RNA from various cells is subjected to this type of salt precipitation. Fig. 3 and Table 1 demonstrate the details of experiments in which the content of double-stranded RNA was determined in HnRNA molecules of various sizes, as well as in polyribosomal mRNA from the cytoplasm. The data indicate that molecules taken from the 100S and 50S regions of a nuclear RNA gradient contain about the same

percentage of the double-stranded RNA (i.e., about 3%). Since the molecules from different regions of the sucrose gradient vary in size over a range of at least 3–5, the constant percentage of double-stranded RNA of the same size distribution (as determined by polyacrylamide gel electrophoresis analysis) in these molecules suggests that at least several regions of double-stranded RNA exist in the larger HnRNA molecules. Although 0.5% of the labeled mRNA was found to be ribonuclease-resistant, no double-stranded RNA was found by gel electrophoresis of the same size distribution as that seen in the HnRNA (Fig. 3*C*). There was, however, in the mRNA a ribonuclease-resistant fraction bound to hydroxyapatite that migrated in the gel close to the dye marker. The nature of this material is presently unknown.

Although all the RNA preparations that were used in previously described experiments came from cells that had been labeled in the presence of a low concentration of actinomycin D to block ribosomal RNA synthesis (25), we felt it was necessary to prove that ribosomal precursor was not the source of the double-stranded RNA. Cells were labeled with [^{3}H]-uridine in the absence of actinomycin, and the purified 45S RNA fraction was tested and found to have at least 5-fold less double-stranded RNA than the extranucleolar HnRNA.

At this point in the investigations we asked whether double-stranded RNA arises: (*i*) because two polynucleotide chains are linked by hydrogen bonds (intermolecular base pairing) or (*ii*) because different regions of the same polynucleotide chain are linked by hydrogen bonds (intramolecular base pairing).

To determine which of these two general classes of possibilities is correct, labeled high molecular weight HnRNA was boiled in a low-salt concentration (15 mM NaCl–1.5 mM Na citrate) and immediately chilled in the presence of a high-salt concentration (0.30 M NaCl–0.030 M Na citrate) before it was incubated with ribonuclease after it was cooled. If the double-stranded regions arose as a result of intermolecular base pairing, the complementary regions should come apart during the boiling and would no longer be detected; if intramolecular base pairing accounts for the double-stranded RNA, the complementary regions would have a chance to reform very rapidly ("snap back"). The results of such experiments show that almost all of the double-stranded RNA in the large HnRNA molecules was recovered after boiling and chilling, followed by 1 min at 37° in the high-salt concentration, suggesting that the double-stranded regions exist as intramolecular loops within large RNA molecules (compare Table 1, Exp. 2*b* with Exp. 1). This is in contrast to the results obtained with the purified double-stranded RNA regions. When material similar to that shown in Fig. 1 was boiled in low salt, cooled in high salt, and then tested in high salt for RNase sensitivity, it was found to be completely digestible by RNase, indicating that the nuclease breakage of large molecules used during the preparation of double-stranded RNA had in some way destroyed the intramolecular linkage responsible for the immediate reassociation seen in the previous experiment.

The spontaneous reassociation of double-stranded RNA was also demonstrated in another type of experiment. [^{3}H]uridine-labeled HnRNA molecules larger than 70 S were precipitated from a sucrose gradient, and a sample was dissolved in a solution of 90% dimethylsulfoxide (Me_2SO)–10% NETS buffer [10 mM Tris·HCl (pH 7.4)–0.1 M NaCl–10 mM EDTA–

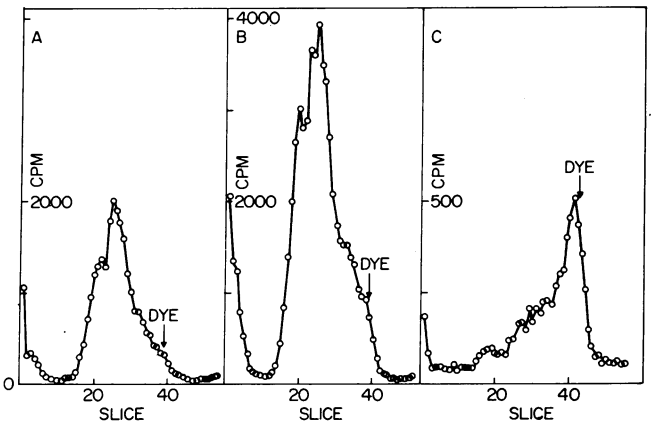

FIG. 3. (*A*) and (*B*) [^{3}H]uridine-labeled nRNA was fractionated into various size classes by sedimentation through a sucrose gradient. The RNA from the 50S and 100S regions of the gradient were collected, and double-stranded RNA was isolated from each. The ribonuclease-resistant hydroxyapatite-bound fraction from each size class was then subjected to electrophoresis in a 15% acrylamide gel. The gels were cut into 2-mm slices and assayed for radioactivity. *Dye* marks the position of the bromphenol blue marker. (A) double-stranded RNA from 100S HnRNA; (B) double-stranded RNA from 50S HnRNA. (*C*) [^{3}H]uridine-labeled mRNA was isolated from polysomes by EDTA release (22), and the ribonuclease-resistant fraction bound to hydroxyapatite was subjected to electrophoresis on a 15% acrylamide gel.

0.2% sodium dodecyl sulfate], a solvent known to denature double-stranded RNA (27). A second portion of these RNA molecules was dissolved in NETS buffer only. Both samples

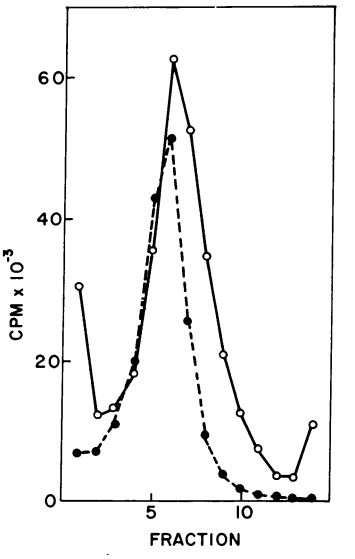

FIG. 4. [^{3}H]uridine-labeled HnRNA larger than 70 S was isolated from a sucrose gradient and concentrated by precipitation with 2 M lithium chloride. Half of the precipitate was dissolved in NETS buffer and the other half in 90% dimethylsulfoxide (Me_2SO)–10% NETS buffer. Both samples were placed at 37° for 20 min and then diluted 10-fold with NETS buffer and centrifuged at 20° through a 15–30% sucrose gradient at 40,000 rpm for 2.5 hr in a Spinco SW40 rotor. Fractions were collected and assayed for acid-insoluble radioactivity. O, double-stranded RNA treated with Me_2SO (3.6%); ●, untreated double-stranded RNA (4.1%). Sedimentation is from right to left. 32S RNA would be found in fractions 11 and 12.

TABLE 2.

Input cpm	Hours	cpm Bound to filter: DNA filter	cpm Bound to filter: Blank	% Input hybridized
5900	2	2770	33	46.5
1280	2	495	41	38.8
5900	10	3750	47	64.5
1280	10	820	32	64.0
5900	24	4120	40	68.0
1280	24	870	38	68.0

Double-stranded RNA denatured by boiling in 0.1 × SSC was hybridized to 40 μg HeLa cell DNA as described (24). Hybridized cpm represent ribonuclease-resistant, filter-bound radioactivity.

were incubated at 37° for 20 min. The sample treated with Me_2SO was then diluted 10-fold with NETS buffer, and both samples were centrifuged through sucrose gradients that did not contain Me_2SO (7) at the same speed for the same length of time as in the original preparation. The gradients were fractionated, and those fractions from each gradient containing the rapidly sedimenting RNA molecules were collected and assayed for double-stranded RNA as previously described. The sedimentation patterns of the radioactive RNA from both Me_2SO-treated and untreated HnRNA are identical (Fig. 4), and the molecules sedimented essentially as before the Me_2SO treatment. In addition, both samples still contained about 3–4% double-stranded RNA of similar size. Apparently, the complementary regions in the sample treated with Me_2SO "snapped-back" when the RNA molecules were centrifuged out of the Me_2SO just as when heat-denatured molecules were chilled in high-salt buffer and incubated for 1 min at 37° (compare, Table 1, Exp. 3 with Exp 2*b*).

A final set of experiments was performed to examine the ability of denatured double-stranded RNA to hybridize to cellular DNA (Table 2). Since double-stranded RNA obtained by nuclease digestion of HnRNA remains denatured after boiling, the hybridization of denatured double-stranded RNA to DNA should not be significantly inhibited by "snap-back" of complementary RNA regions (i.e., RNA–RNA hybridization). As much as 50% of the denatured double-stranded RNA was hybridized within 6 hr to DNA, while about 70% of the denatured double-stranded RNA was hybridized after 24 hr. The condition used for these hybridization studies were the same as previously used to define "rapidly hybridizing" regions of heterogeneous nRNA. The previous work showed that a class(es) of rapidly hybridizing nRNA constitutes 10–15% of the total HnRNA molecule (27, 28). Since the double-stranded RNA represents about 3% of the total HnRNA and a large amount of it hybridizes rapidly to DNA, it is possible that a sizeable fraction of the HnRNA sequence that hybridizes rapidly to DNA may occur in HnRNA as double-stranded regions.

DISCUSSION

Progress in the study of the relationship between HnRNA and mRNA has been slow in the past because diagnostic characteristics of either class of molecules were not known (5). This accounts for the activity triggered by the finding that both HnRNA and mRNA contain poly(A) (9–13, 30). The purpose of the experiments reported here was to explore whether the double-stranded cellular RNA reported by many workers exists in either HnRNA or mRNA, and if so, whether it might provide another biochemical marker for examining the relationship between HnRNA and mRNA.

The results show that about 3% of HnRNA molecules are in an RNase-resistant form with properties of double-stranded RNA. Furthermore, the evidence indicates that these double-stranded regions occur intramolecularly in HnRNA molecules. The double-stranded regions hybridize rapidly to cellular DNA and, thus, it may be inferred that they are transcribed from DNA sequences that are repeated many times in the genome (31). Since all large HnRNA molecules have rapidly hybridizing sequences (29), it becomes likely that most also have double-stranded regions. The foregoing summary lends considerable significance to the finding that the double-stranded regions are not found in mRNA. mRNA has a lower content than does HnRNA of the most rapidly hybridizing sequences, a finding consistent with the absence of double-stranded RNA in these molecules (29).

Before these findings will have greater value in relating HnRNA to mRNA, the distribution of the double-stranded RNA regions within HnRNA needs further exploration. To begin with, the frequency of such regions within HnRNA cannot be accurately estimated at the moment because the correct size of the double-stranded regions is not known. If HnRNA with a 40–70S sedimentation rate is taken to be 10,000–20,000 nucleotides long (32) and the base composition of HnRNA and double-stranded RNA are both about the same, a figure of 3% as double-stranded RNA indicates that about 300 nucleotides per 10,000 are in double-stranded regions. The breadth of the double-stranded RNA peak in 15% acrylamide gels indicates that double-stranded RNA regions of different size exist in our preparations. (A single size species of RNA would be found in only 3–4 gel slices.) Kimble and Duesberg report that double-stranded RNA from HeLa cells sediments at 7–11 S, which would correspond to about 500–1500 nucleotides (judging from the known size of reovirus double-stranded RNA). We have not observed such large fragments regardless of the digestion conditions used.

The question must remain open for the moment as to whether the double-stranded regions exist once every 10,000–20,000 nucleotides, i.e., one per HnRNA molecule, or one every 1000–2000 nucleotides, i.e., once per mRNA length.

Another very important point is whether the complementary regions exist immediately adjacent to one another or whether they are separated by relatively long noncomplementary regions of HnRNA. Which of these two possibilities is correct can be determined by breaking RNA molecules before denaturation to determine the size of the RNA below which immediate renaturation ("snap-back") fails to occur.

A note of clarification and caution should be added in closing. While the use of the double-stranded regions in mapping the topology of the HnRNA seems very promising, there is at the moment no proof that these intramolecular double-stranded regions exist as such inside the cell. They have the capacity to anneal within a molecule very rapidly, and thus may exist only in the purified RNA preparations. That they seem to come from repeated regions of the genome suggests that they may have something to do with regulation of genetic expression. However, they may never function intracellularly in the double-stranded form.

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