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An Adenylic Acid-Rich Sequence in Messenger RNA of HeLa Cells and Its Possible Relationship to Reiterated Sites in DNA

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ABSTRACT **Messenger RNA from HeLa cells contains, as part of the polynucleotide chain, RNase-resistant sequences that are labeled by adenosine but not by uridine. Heterogeneous nuclear RNA also contains adenylate-rich RNase-resistant regions, but in lower proportion than messenger RNA. Hybridization to DNA of ^{32}P-labeled messenger RNA reveals that some of the adenylate-rich region is included in the rapidly-hybridizing fraction.**

The experiments of Britten and Kohne (1), Walker (2), McCarthy (3), and others (4, 5) have emphasized in recent years that the nuclear DNA from eukaryotic cells contains similar but nonidentical sequences which are repeated many times in the haploid genome. It has been argued that even though some classes of proteins have many members, the frequency of repetition for some DNA sequences exceeds reasonable expectations of the size of families of proteins. Accordingly, it has been proposed (6) that these repeated sequences in eukaryotic DNA probably have some "regulatory" role.

In nucleic acid hybridization experiments, the repeated sequences are recognized by their relatively greater rate of interaction than sequences which appear only once or a few times per genome. Many of these so-called "reiterated regions" of the DNA are apparently transcribed into RNA (5, 7–9). Both the HnRNA (heterogenous nuclear RNA), almost all of which turns over in the cell nucleus (10), as well as the mRNA, which functions on cytoplasmic ribosomes (11), contain regions which attach to reiterated regions of the DNA as RNase-resistant hybrids (7). Because of the fact that mammalian cells devote such a large amount of their RNA synthetic potential to the synthesis of HnRNA, most of which has been shown not to participate in protein synthesis, it has also been frequently suggested that HnRNA or some parts of it may play a "regulatory" role. The recent finding that each HnRNA molecule contains at least one rapidly hybridizing sequence (i.e., complementary to reiterated DNA), as do most (if not all) messenger RNA molecules, is consonant with a proposed regulatory role (12). The difficulties in critically examining either the hypothesis that reiterated sequences in DNA or that mRNA or HnRNA sequences play regulatory roles are that (*a*) virtually no somatic cell genetics exists and therefore no "regulatory" mutants exist in cells in which studies on reiterated nucleic acids have been performed, and (*b*) no sufficiently diagnostic biochemical makers in the reiterated nucleic acid molecules have previously been available to allow an inquiry into their function.

Abbreviations: HnRNA, heterogeneous nuclear RNA; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl–0.015 M sodium citrate.

Two recent findings prompted the present work, the aim of which was to determine if chemical differences existed between the rapidly-hybridizing RNA sequences from the HnRNA and mRNA. First, it has been shown that HnRNA from cells labeled with [^{3}H]uridine contained sequences which hybridized more rapidly than any uridine-labeled cytoplasmic mRNA sequences (12). When it was reported that purified hemoglobin mRNA from reticulocytes (13), HnRNA from Ehrlich ascites tumor cells (14), and RNA produced by vaccinia cores (15) contained a relatively RNase-resistant region that was very rich in adenylate, we decided to examine the rapidly-hybridizing HnRNA and cytoplasmic RNA sequences from HeLa cells to determine if they were chemically related and if either was adenylate-rich. These experiments have demonstrated that both mRNA and HnRNA from growing cells contain an adenylate-rich RNase-resistant portion and that the rapidly hybridizing mRNA fraction is unusually rich in adenylate while the rapidly-hybridizing HnRNA has a "balanced" base composition.

METHODS AND MATERIALS

The growth of HeLa cells in suspension culture, cell fractionation, RNA extraction from nuclei and polyribosomes, DNA preparation from purified nuclei, and hybridization procedures have been previously detailed (7, 16). Briefly, the following should be noted for the experiments in this paper. Three labels were employed for RNA: uridine, adenosine, and ^{32}P. In all cases, RNA was prepared from cells after 3–4 hr of incubation at a low concentration (0.05 μg/ml) of actinomycin D so that no labeled rRNA or ribosomal precursor RNA was present in the samples (17). Polyribosomes were isolated from the cytoplasm by zonal sedimentation and mRNA was released by EDTA treatment (11). Subsequent isolation of labeled mRNA–protein complexes was accomplished by a second zonal separation as previously described (12, 18). Base composition was determined at pH 3.5 by high-voltage paper electrophoresis of alkaline hydrolysates of ^{32}P-labeled RNA (19). The pancreatic RNase used both in the hybridization assay and in the test for ribonuclease resistance was purchased from Sigma and the T_1 ribonuclease from Calbiochem. Both were heated to 80°C for 15 min in 0.05 M acetate, pH 5.1, to destroy DNase.

RESULTS

In order to enquire into the presence and nature of RNase-resistant sequences in mRNA from HeLa cells, we prepared polyribosomes from cells labeled in their mRNA with either adenosine or uridine, as described in *Methods*. Labeled mRNA

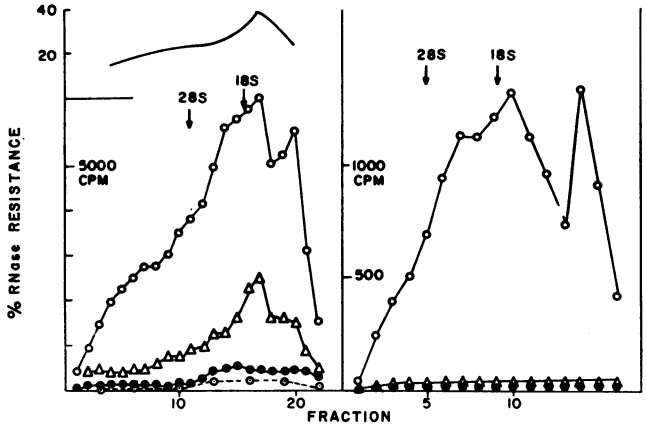

FIG. 1. mRNA from HeLa cells labeled with adenosine or uridine. 1.6×10^8 HeLa cells were suspended for 25 min in 30 ml of growth medium containing 1.5 μg of actinomycin D and then labeled by the addition of [^{3}H]adenosine (8 Ci/mmol; 10 mCi in 10 ml of medium) for 3 hr. Another sample of 0.8×10^8 cells was suspended for 25 min in 15 ml of medium with 0.75 μg of actinomycin D and then labeled by the addition of [^{3}H]uridine (28 Ci/mmol; 5 mCi in 5 ml of medium) for 3 hr. Polyribosomes were prepared from the post-mitochondrial supernatant by zonal sedimentation (11) and RNA was released by SDS (final concn 0.5%), ethanol-precipitated, redissolved, and subjected to a second zonal sedimentation in sucrose gradients (40 rotor, 16 hr, 23,000 rpm, 28°C in 15–30% sucrose (w/w) dissolved in 0.01 M Tris–0.1 M NaCl–0.05% SDS). Fractions of about 0.5 ml were collected and radioactivity in 0.05-ml samples was assayed. —○—, cold-acid-precipitable; —△—, dilution with 2 ml of 2 × SSC, containing RNase, 1.6 μg/ml followed by 30 min incubation at 37°C, and cold acid precipitation; —●—, dilution with 2 ml of 0.1 × SSC containing RNase, incubation, and precipitation as above; —⊙—, dilution into 0.3 N KOH followed by incubation as 37°C for 18 hr before precipitation.

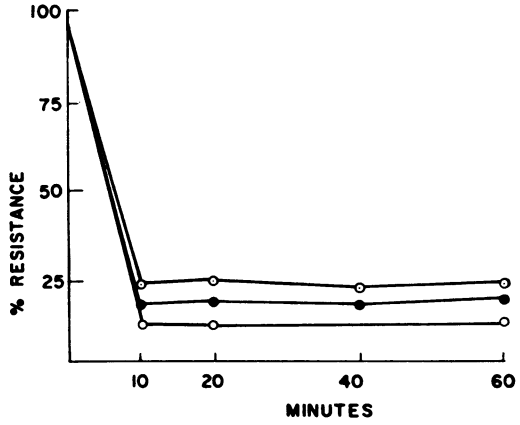

FIG. 2. Adenosine-labeled mRNA of various sizes was collected by ethanol precipitation of selected fractions from a sucrose gradient (see Fig. 1), redissolved in 0.5 × SSC, and digested at 37°C with 1.6 μg/ml of pancreatic RNase for the indicated times. —○— 28S mRNA; —●— 24S mRNA; —⊙— 18S mRNA.

was released from the polysomes either with sodium dodecyl sulfate (SDS) and re-examined by sucrose gradients or released by EDTA treatment followed by re-sedimentation of ribonucleoprotein particles through sucrose gradients. Fig. 1 shows the sedimentation profile of SDS-released mRNA. Samples from the sucrose gradient were subjected to cold acid precipitation and alkali hydrolysis or diluted and exposed to RNase in the presence of 0.3 M NaCl–0.03 M sodium citrate (2 × SSC, standard saline citrate) or 0.1 × SSC. The results demonstrate that adenosine-labeled RNA contained a fraction that was RNase-resistant at high ionic strength but completely destroyed by alkali and largely destroyed by RNase at low ionic strength. Electrophoresis of alkaline hydrolysates of the adenosine-labeled RNA revealed that even after 3 hr of labeling over 90% of the label was in adenylic acid. No such RNase-resistant fraction was found in uridine-labeled mRNA. In addition, it appeared that the larger the mRNA (the higher the sedimentation rate) the lower the proportion of RNase resistance.

Since the RNase digestions in the experiment of Fig. 1 were performed with a single digestion time and salt concentration, additional experiments were performed to test the RNase resistance in differently-sized adenosine-labeled HnRNA and mRNA molecules and in HnRNA after several digestion times and in different ionic strengths. Fig. 2 confirms that the RNase-resistant adenosine-labeled RNA was present in proportionately larger amounts in smaller mRNA than in larger mRNA.

The experiments of Fig. 3 examined the RNase resistance of both adenosine- and uridine-labeled mRNA and HnRNA at various ionic strengths. Both mRNA and the high molecular weight HnRNA (larger than 35S) labeled with either adenosine or uridine were almost completely digested to acid solubility (more than 96–99%) in very low ionic strength (0.1 × SSC), while all the fractions except the uridine-labeled mRNA showed considerable RNase resistance in 2 × SSC. Although HnRNA labeled with uridine is more than 98% sensitive to RNase in moderate salt concentration (0.5 × SSC), about 4–5% of the adenosine-labeled HnRNA resists digestion at this salt concentration. (The actual figures for the experiment plotted in Part *B* of Fig. 3 were 4.1% resistance for adenosine-labeled HnRNA and 1.1% resistance for uridine-labeled HnRNA.) The mRNA was about 15–20% resistant to digestion at this salt concentration.

The RNase resistance in adenosine-labeled mRNA appeared to be in the mRNA itself and due to an intramolecular structure, because boiling, quick cooling, and RNase digestion of a sample identical to the one plotted in Fig. 3 did not change the results at all. In addition, another mRNA sample from the 20–25S region of zonal separation of adenosine-labeled mRNA was treated with $(CH_3)_2SO$ (95%), then diluted in H_2O and re-examined by sucrose gradient sedimentation (Fig. 4). This procedure should have denatured any double-stranded nucleic acid and was used as a test for whether the RNase-resistant region was an integral part of the mRNA polynucleotide chain (21). Although the RNA did not sediment as homogeneously after $(CH_3)_2SO$ treatment, probably because of breaks in the RNA chains introduced during the experimental procedures, the same proportion of RNase resistance was still found in the 20–25S material.

The sedimentation profile of the RNase-resistant portion from a digest of adenosine-labeled RNA was examined next. Different mRNA preparations were digested by RNase in 0.5 × SSC, phenol-extracted, and examined by sucrose gradient sedimentation. Regardless of the nature of the starting

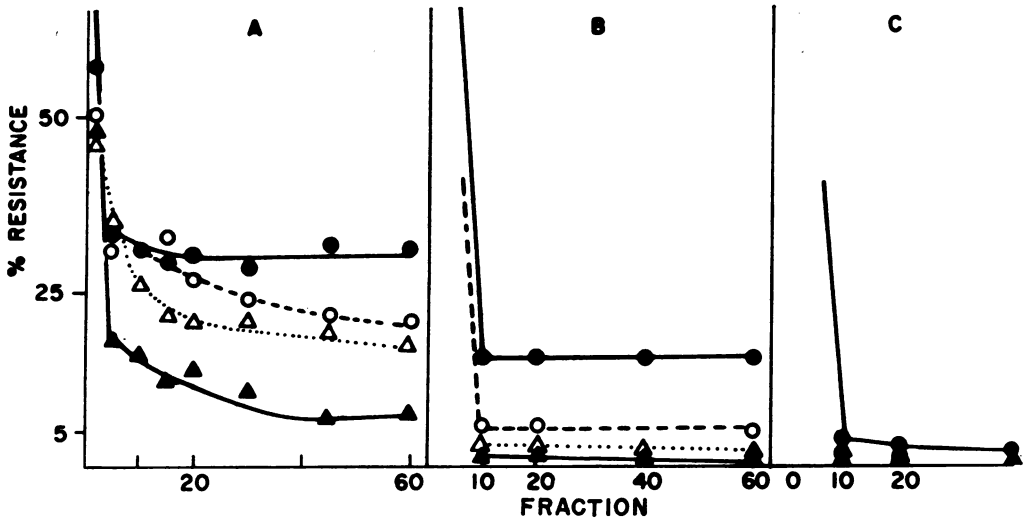

FIG. 3. RNase sensitivity of varous types of HeLa cell RNA. HnRNA (>45S) or mRNA labeled either with adenosine or uridine were prepared and exposed to RNase, 1.6 μg/ml in 2 × SSC (*A*), 0.5 × SSC (*B*), or 0.1 × SSC (*C*). HnRNA, adenosine-labeled —○—; uridine-labeled —△—; mRNA, adenosine-labeled —●—, uridine-labeled —▲—.

mRNA sample (Fig. 5), the RNase-resistant portion was found to be very similar in size, sedimenting about 10% faster than 4S transfer RNA from *E. coli*.

The experiments described thus far indicate that at least some mRNA molecules contain an RNase-resistant adenosine-labeled region, which appears to be about 100–200 nucleotides long. The relationship of this region to the nuclear DNA was investigated by hybridization experiments.

mRNA and HnRNA from ^{32}P-labeled cells were prepared and exposed to filter-bound HeLa-cell DNA for various periods of time. After treatment with RNase, hybridized RNA was recovered (12). Both the RNase-resistant hybrids and the starting RNA were subjected to alkaline degradation and base-composition analysis (Table 1). The base composition of the HnRNA labeled at low actinomycin concentration was shown to be similar to that previously found (22) for cells not treated with actinomycin, namely cytosine (C) 24.1%, adenine (A) 24.1%, guanine (G) 20.5%, and uridine (U) 31.1%. The HnRNA that hybridized within 0–3, 0–6, or 6–24 hr was quite similar in base composition to the total

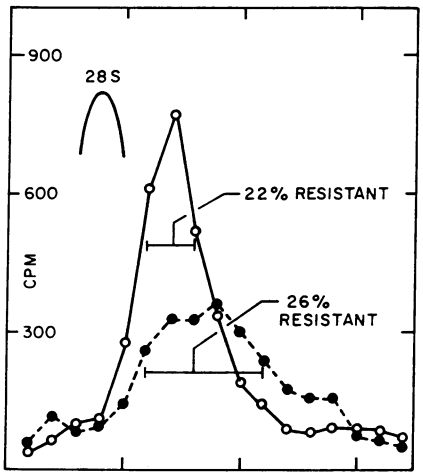

FIG. 4. Adenosine-labeled mRNA (Fig. 1) was precipitated from the 25S region of a sucrose gradient and redissolved either in SDS buffer (0.01 M Tris (pH 7.4)–0.1 NaCl–0.2% SDS) or in 90% dimethyl sulfoxide (Fisher). Each sample was then diluted with 2 volumes of SDS buffer and centrifuged again as in Fig. 1. The radioactivity peaks were precipitated, redissolved in 2 × SSC, and tested for RNase resistance.

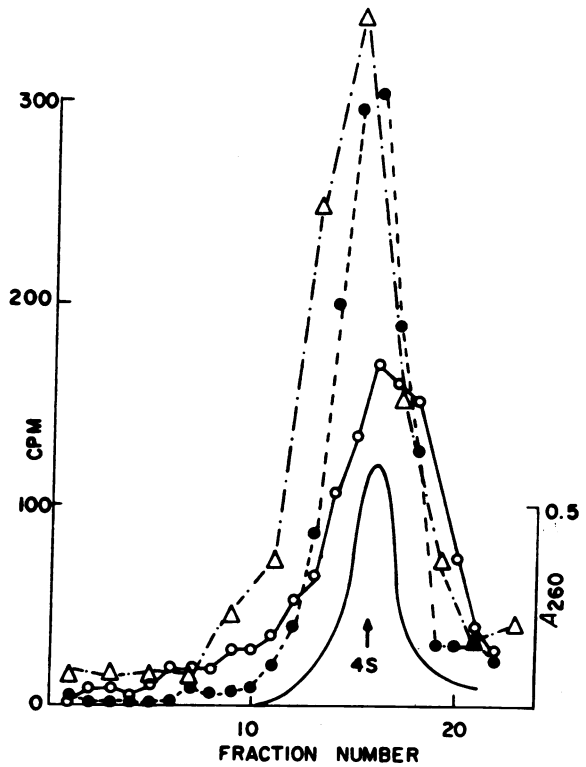

FIG. 5. Samples of mRNA of various sizes were digested with RNase (1 μg/ml, 37°C, 30 min), after which phenol extraction and zonal sedimentation in sucrose–SDS was carried out at 40,000 rpm for 21 hr at 28°C. A sample of *E. coli* transfer RNA (kind gift of Dr. M. Gefter) was used as an absorbance marker in a separate tube. Acid-precipitable radioactivity was then determined on samples of the gradients. Starting mRNA of 15S —△—; 25S —○—; mixture of all sizes of mRNA —●—.

TABLE 1. *Base composition of mRNA, HnRNA, and RNA·DNA hybrids formed by each*

RNA sample	C	A	G	U	G + C
HnRNA	24.1	24.1	20.5	31.1	44.6
HnRNA hybrid					
0–3 hr	30.0	26.3	20.2	23.4	50.2
0–6 hr	28.6	25.1	19.4	26.9	48.0
0–6 hr	22.9	27.4	21.9	27.1	44.8
6–24 hr	25.3	28.3	21.9	25.3	47.2
mRNA	24.4	30.1	21.8	23.8	46.2
mRNA hybrid					
0–6 hr	18.7	44.0	17.9	19.4	36.6
0–6 hr	17.8	51.3	16.8	14.1	34.2

^{32}P-labeled HnRNA or mRNA was hybridized to a series of DNA filters containing 40 μg per filter for 3 or 6 hr. The HnRNA remaining in the supernatant was further hybridized from 6 to 24 hours. RNase-resistant hybrids (0.3–1% of input; total cpm in various samples from 5000 to 50,000) were recovered and base composition was determined after alkaline hydrolysis.

HnRNA. The mRNA had an overall base composition of C 24.4%, A 30.1%, G 21.8%, U 23.8%, which was similar to values in previous reports (11, 23). The hybrid from the mRNA, however, was enriched in adenosine from 44% to 51%.

DISCUSSION

The experiments of this paper bear on two difficult but pressing problems in the study of mammalian cell nucleic acids: the relationship between HnRNA and mRNA, and the nature and possible role of repeated sequences in eukaryotic DNA.

Since the discovery of the similar DNA-like base compositions of HnRNA and mRNA, the hypothesis has been considered (22–25) that mRNA is fashioned from a higher molecular weight nuclear precursor. Decisive experiments for or against this proposal are still lacking. One suggestive recent experiment in favor of this pathway of mRNA formation comes from the study of the nuclear RNA products of cells in which SV40 viral DNA has been integrated (26). In such cells, HnRNA molecules of very high molecular weight ($> 4 \times 10^6$) were shown to contain viral specific RNA sequences, while polysomal viral specific mRNAs were much smaller. The present result that adenylate-rich sequences are present in both HnRNA and mRNA molecules again indicates the possibility that at least some mRNA may arise from specific nuclear processing of HnRNA. The recent experiments of Edmonds and Caramela (14) and Edmonds, Vaughan, and Nakazato (27) are quite suggestive in this regard because they have shown that the adenylate-rich sequences from HnRNA and mRNA of HeLa cells are comparable in size (about 200 nucleotides long) and in base composition (more than 90% adenylate in both cases).

No evidence exists as to the location of the adenylate-rich region within the mRNA molecule. Since it appears to be present in many mRNA molecules which act as monocistronic messengers (28) coding for many different proteins, the adenylate-rich region is probably not itself translated and would therefore probably be terminal. Thus, it is hard to escape the conclusion that the adenylate-rich region is a signal related most probably to translation or mRNA formation and transport.

The second aspect of the present work concerns the base composition of rapidly-hybridizing RNA. Because these hybridized sequences interact with DNA within a few hours while the majority of the RNA remains unannealed, it is assumed that the "reiterated" DNA sites are responsible for the observed hybridization. When the base composition of the hybrids from HnRNA was examined, no significant change in overall base composition was detectable between bound and unbound molecules, which indicates that even if the adenylate-rich RNA in HnRNA were hybridizing, it was a minority of the hybrid. A different result was obtained for the rapidly-hybridizing sequences from the mRNA. Here, over half of the nucleotides in the total "hybrid" were adenylic acid. These results definitely indicate that the rapidly hybridizing sequences in mRNA either include the adenylate-rich region as a true hybrid or that sequences that are neighbors to the adenylate-rich region are the rapidly hybridizing sequences. The unpaired RNA in the hybrid test is removed by nuclease digestion at 0.3 M NaCl (16), and unpaired regions rich in adenylate would not necessarily be digested at the salt concentration (29). Thus it is not possible to decide on the basis of the present evidence whether the adenylate-rich region is transcribed from the DNA or whether it is added on next to a reiterated sequence which then becomes responsible for the inclusion of the adenylate-rich region in the hybrids formed by mRNA. This hypothetical addition of adenylate-rich [or poly(A)] sequences might be catalyzed by enzymes of the type isolated by Edmonds and Abrams from thymus cell nuclei (30).

In thinking about DNA primed synthesis versus sequences added after transcription, it should be pointed out that the inhibition by actinomycin (14) of the appearance of the adenylate-rich region in either HnRNA or mRNA may not reflect inhibition at the level of transcription, but a decrease in the substrate for the potential attachment of adenylate-rich sequences.

In any event it appears that the reiterated sites in mRNA must play some role other than that of structural gene transcript, since they either include or are adjacent to the adenylate-rich region. Whether the rapidly hybridizing HnRNA sites that do not include the adenylate-rich region are really different from the rapidly-hybridizing mRNA sites will depend on whether the DNA truly encodes the adenylate-rich region.

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