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Polyadenylic Acid Sequences in the Heterogeneous Nuclear RNA and Rapidly-Labeled Polyribosomal RNA of HeLa Cells: Possible Evidence for a Precursor Relationship

(messenger RNA/poly(dT)-cellulose)

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ABSTRACT **Polyadenylate sequences have been found covalently linked in heterogeneous DNA-like nuclear RNA of HeLa cells. This poly(A) material seems homogeneous in size and accounts for about 0.5% of such RNA. Similar poly(A) sequences were found in rapidly-labeled polyribosomal RNA, thought to be messenger RNA. A possible model for mRNA synthesis from large heterogeneous nuclear RNA precursor molecules is discussed.**

The synthesis of a relatively homogeneous population of polyriboadenylic acid [poly (A)] sequences of 150–200 nucleotides in the nucleus of Ehrlich ascites cells was recently described (1). The suppression of this synthesis with actinomycin D suggested that AMP-rich sequences were transcribed on DNA. Knowledge of the precise location of these sequences with respect to those RNA species which characterize eukaryotic cells was required to interpret these observations. Suspension cultures of HeLa cells were selected for such studies, not only for the techniques available for the isolation of well-characterized subcellular components (2) and RNA species (3, 4), but particularly for the detailed knowledge, available from kinetic analyses, of the flow of radioactivity through and into various RNA species of these cells (5).

The poly(A) content of RNA fractions from the nucleus and cytoplasm of HeLa cells has been measured by a technique dependent on the hydridization of poly(A) sequences to polydeoxythymidylate oligomers covalently bound to cellulose (6). Highly purified poly(A) can be quantitatively recovered from suitably prepared RNA samples on temperature-controlled filters of poly(dT)-cellulose (7).

We show here a broad distribution of poly(A) sequences within two classes of RNA that are themselves characteristically heterogeneous, i.e. the rapidly labeled polydisperse RNA (HnRNA) of the nucleus, and the polyribosome-associated RNA with the sedimentation characteristics of messenger RNA (mRNA). The poly(A) sequences recovered from all size classes of both types of RNA are rather homogeneous and show similar electrophoretic mobilities in acrylamide gel that correspond to an approximate length of 150–200 nucleotides.

Evidence is presented that the poly(A) sequences detected after ribonuclease digestion of the HnRNA are in fact covalently bonded within that RNA. Equally compelling evidence for the covalent linkage of poly(A) detected in mRNA to that RNA has not yet been obtained, although such a linkage is considered likely.

The implications of the detection of poly(A) sequences of similar length in both cytoplasmic mRNA and nuclear HnRNA will be considered with respect to a precursor–product relationship proposed (8) to link these RNA species.

Abbreviations: HnRNA, rapidly labeled polydisperse nuclear RNA ("heterogeneous nuclear RNA"); SSC–SDS, 0.15 M NaCl–0.015 M sodium citrate–1% (w/v) sodium dodecyl sulfate.

MATERIALS AND METHODS

Cell culture

Suspension cultures of HeLa cells were maintained as previously described (9), at cell concentrations of 2–5 $\times$ 10^5 cells/ml.

Cell labeling and fractionation

Cells were concentrated to 3 $\times$ 10^6 cells/ml for labeling with $^{32}PO_4$ (0.2 mCi/ml) in phosphate-free Eagle's minimal essential medium, supplemented with 5% normal calf serum. After the labeling, the cells were pelleted and rinsed with ice-cold Earle's salts solution, swollen in hypotonic medium, broken by Dounce homogenization, and fractionated by centrifugation (2, 3). Except where noted, the detergent rinsing of nuclei was omitted in order to facilitate extraction of total nuclear RNA. All RNA extractions were done by the hot phenol–sodium dodecyl sulfate method (4).

Polyribosomes were analyzed by sucrose gradient centrifugation of combined cytoplasmic fractions (10), with or without the addition of Brij-58 (nonionic detergent) and sodium deoxycholate, as specified.

RNA analysis

Sedimentation analysis and acrylamide gel electrophoresis (11) of RNA, and base composition analysis (12) of RNA hydrolysates, were as described. All radioactivity measurements were made by scintillation counting in Omnifluor (New England Nuclear)–toluene solution, with addition of NCS reagent (Amersham-Searle) for trichloroacetic acid-precipitated gradient fractions or Triton X-100 (Rohm and Haas) for gel fractions collected by the Maizel fractionator.

Polyadenylic acid analysis

Poly(A) was detected and quantitated essentially as previously described (1). RNA in 2.0 ml of 0.03 M Tris (pH 7.5)–0.002 M $MgCl_2$, mixed with an internal standard of [^{3}H]-poly(A) (0.025 μCi in 50 μg, Miles Laboratories) was digested

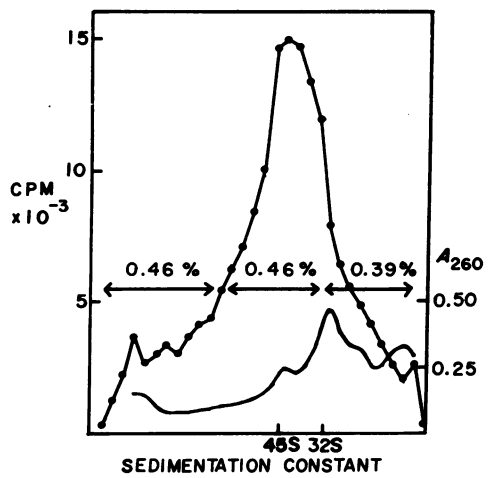

FIG. 1. Sedimentation analysis and poly(A) content of total nuclear RNA after 60-min labeling with $^{32}PO_4$. Poly(A) content is shown as % of total labeled RNA. The RNA was analyzed by sedimentation on a 15–30% sucrose gradient in NETS (0.2% SDS–0.1 M NaCl–0.01 M EDTA–0.01 M Tris, pH 7.4) for 14.5 hr at 19,000 rpm, 18°C, in the SW40 rotor of the Beckman ultracentrifuge. Fractions were divided into portions for trichloroacetic acid precipitation and determination of poly(A) contents. A_{260} is shown in the lower curve.

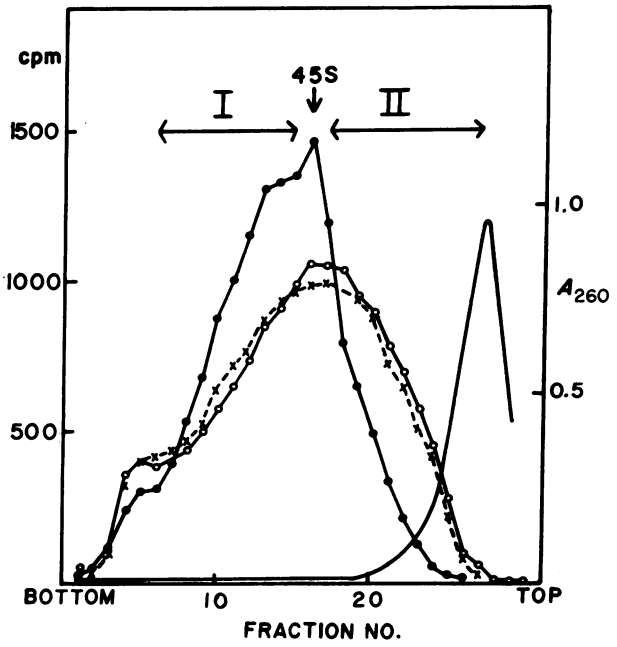

FIG. 2. Competition experiment showing that poly(A) is not adventitiously bound to large (>45S) HnRNA. $^{32}PO_4$-labeled HnRNA with $s > 45$ S was prepared as in the experiment of Fig. 1. The RNA was divided into three equal portions, of which one (O—O) was denatured by dissolving it in 0.4 ml of Me_2SO containing 1 mM EDTA. After 10 min at 37°C, 0.6 ml of $^1/_6 \times$ SSC–SDS (SSC–SDS = 0.15 M sodium chloride–0.015 M sodium citrate–1% SDS) was added. A second portion (×—×) was similarly treated except that the $\times$ $^1/_6$ SSC–SDS solution also contained a 10-fold excess, relative to the [^{32}P]RNA, of nonradioactive large HnRNA (>45S) which was fragmented by heating for 20 min at 100°C in the SSC–SDS solution. The third portion of RNA (●—●) was directly dissolved in 0.4 ml of 1 mM EDTA to which was added 0.6 ml of $^1/_6 \times$ SSC–SDS solution. All three samples were then analyzed by sedimentation as described for Fig. 1. The radioactivity profiles for aliquots from all three gradients are shown, together with the UV-absorption profile for the sample to which excess fragments of HnRNA had been added. The gradients were divided into pools of rapidly (I) and slowly (II) sedimenting RNA for poly(A) analysis, shown in Table 1.

with 10 μg of DNase I (Worthington Biochemicals, DPFF), 100 units of RNase T_1 (Sankyo Preparation from Calbiochem, Corp.) containing 50 μmol of $(NH_4)_2SO_4$, and 0.1 μg of RNase A (Preparation XII, Sigma Biochemicals). After 15 min at 37°C, the digest was cooled to 0°C and made 0.1 M in NaCl and 0.005 M in EDTA. 125 mg of poly(dT)-cellulose (cellulose covalently linked to short oligomers of polythymidylic acid) was then stirred in. After 15 min the suspension was applied to a layer of poly(dT)-cellulose on a temperature-controlled filter. The filter was washed with 0.10 M NaCl at 2°C and 23°C, and bound poly(A) was eluted with 0.01 M Tris, pH 7.5, at 23°C for precipitation either with trichloroacetic acid or with ethanol. All yields are corrected for the recovery of [^{3}H]poly(A), which was 80–90% in these experiments.

RESULTS

Poly(A) sequences in the nucleus

Poly(A) sequences were not found in the nucleolar pellet recovered by zonal sedimentation of homogenates prepared by lysis of nuclei with DNase in high salt buffers (3). This eliminated ribosomal RNA and its precursors as sites of poly(A) sequences and simplified the interpretation of the distribution of poly(A) sequences in the total nuclear RNA (Fig. 1) after sedimentation in a sucrose gradient. Allowing for the large 45–32S peak of radioactive ribosomal precursor RNA, the distribution of poly(A) sequences (shown as percent of total labeled RNA) appeared to parallel that of the HnRNA (not shown), which is characterized by its polydispersity, DNA-like base composition, and rapid rate of turnover. This RNA, which accounts for a large fraction of the total RNA synthesized, turns over so rapidly that it represents only 1 or 2% of the total RNA of the cell (13).

The poly(A) sequences recovered from all size classes of HnRNA showed marked similarities in electrophoretic mobility on polyacrylamide gels. This mobility relative to those of some well-characterized small marker RNA species was similar to that of poly(A) derived from the total nuclear RNA, shown in Fig. 5, which corresponds to a length of about 150–200 nucleotides (14).

This uniformity of size would not support the concept of a heterogeneous nuclear poly(A) population that includes a high percentage of very large poly(A) molecules, but suggests rather that HnRNA molecules contain a covalently linked poly(A) sequence released by the ribonuclease treatment included in the hybridization assay.

A competition experiment (Fig. 2) was designed to rule out the possibility that the small poly(A) molecules are adventitiously bound to HnRNA. In this experiment, HnRNA was denatured in the presence of a large excess of unlabeled fragments of the same HnRNA. Labeled HnRNA molecules with $s > 45$ S were selected for this experiment since they could be separated from nonradioactive fragments of HnRNA by sedimentation velocity. The labeled 50–90S HnRNA was pooled from a gradient analysis similar to that of Fig. 1 and divided into three equal parts. One part was re-sedimented on a similar gradient (●—●), another was re-sedimented after denaturation in dimethyl sulfoxide, and the third was re-sedimented after denaturation in Me_2SO in the presence of a 10-

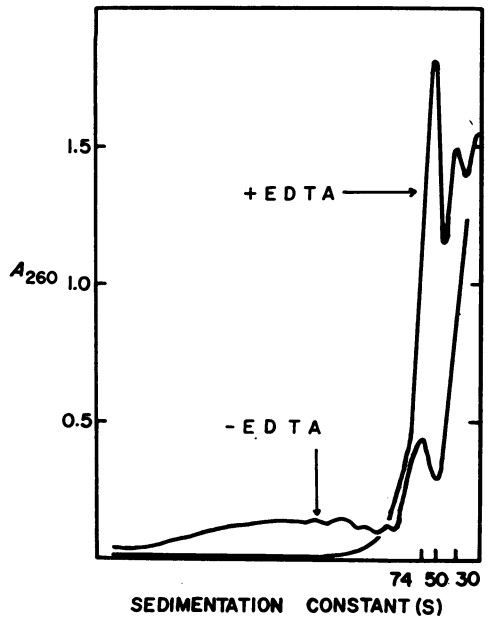

FIG. 3. Sedimentation of cytoplasmic extracts treated or untreated with EDTA to displace mRNA from polyribosomes. A 20-ml culture of HeLa cells at 4×10^6 cells/ml was labeled with $^{32}PO_4$ for 60 min. The cells were then harvested and fractionated. Cytoplasmic extract was prepared and made 0.5% in both Brij-58 and sodium deoxycholate. EDTA was added (to 0.01 M) to half the extract and both samples were sedimented on RSB (0.01 M NaCl–0.001 M $MgCl_2$–0.01 M Tris, pH 7.4) sucrose gradients for 110 min at 25,000 rpm, 4°C, in the SW27 rotor of the Beckman centrifuge. Polyribosome ($s > 74$ S) and nonpolyribosomal regions were separately pooled and analyzed for poly(A), as shown in Table 2.

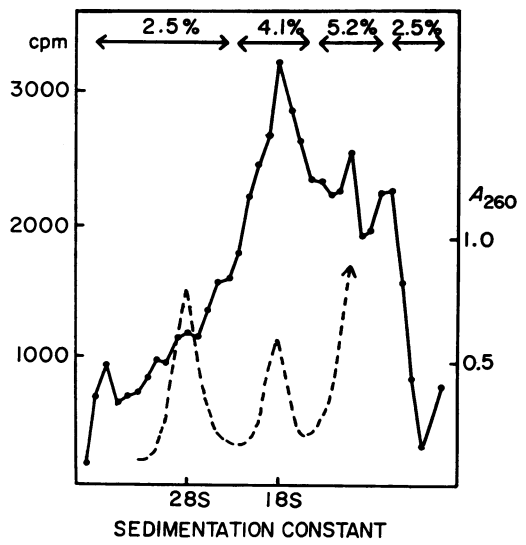

FIG. 4. Sedimentation and poly(A) analysis of rapidly-labeled polyribosomal RNA (mRNA). Polyribosomes were prepared from a culture labeled with $^{32}PO_4$ for 30 min. RNA was extracted from the polyribosomes and run on a sucrose gradient in NETS (see Fig. 1) in the SW40 rotor for 18.5 hr at 24,000 rpm, 18°C. Aliquots of gradient fractions were taken for trichloroacetic acid precipitation and selected fractions were pooled for poly(A) analysis (percentages shown at top). ---, A_{260}.

fold excess of unlabeled HnRNA fragmented by boiling. The A_{260} profile included in Fig. 2 shows that the fragments do not cosediment appreciably with any of the re-sedimented ^{32}P-labeled HnRNA samples.

The untreated, re-isolated HnRNA and the denatured samples (Fig. 2) showed only small reductions in sedimentation constant compared to the original 50–90S. The poly(A) contents of the RNA in the top (II) and bottom (I) half of each gradient are shown in Table 1.

Although the conditions for denaturation in Me_2SO were more than sufficient to melt poly(G)·poly(C) complexes (15), poly(A) was not transferred from the large HnRNA to the small fragments sedimenting in the top half (II) of the gradient. The poly(A) content of this large HnRNA is essentially identical, with or without re-isolation or Me_2SO denaturation, with that of the HnRNA of the experiment shown in Fig. 1.

TABLE 1. *Poly(A) content of large heterogeneous nuclear RNA after denaturation**

Sample	% total cpm in poly(A)	
	I (large RNA)	II (small RNA)
1. Control	0.49	0.93
2. Me_2SO-treated	0.49	0.53
3. Me_2SO-treated, with HnRNA fragments	0.46	0.64

* Details of the experiment are described in Fig. 2.

Similar attempts to remove poly(A) from the large HnRNA by the addition of a large excess of either unlabeled synthetic poly(A) or poly(U) prior to the extraction of nuclear RNA were also unsuccessful. Such competition experiments with poly(U), however, required a modification of the poly(T)-cellulose hybridization assay in order that poly(A) could be detected in the presence of large quantities of poly(U) (unpublished observations).

Since adventitious binding of poly(A) is effectively ruled out by these experiments, a covalent attachment of these sequences to the large HnRNA provides the most satisfactory explanation of the data.

Poly(A) sequences in cytoplasm

A major fraction of the poly(A) sequences detected in the cytoplasm of cells labeled for 1 hr with $^{32}PO_4$ was found in the polyribosome fraction of cytoplasmic extracts sedimented through sucrose gradients.

Fig. 3 shows the distribution of polyribosomes in sucrose gradient analysis with and without prior addition of EDTA. Table 2 shows that almost 90% of the poly(A) sequences sedimented more slowly than polyribosomes when the latter were

TABLE 2. *Displacement of poly(A) sequences from polyribosomes by EDTA*

Treatment of cytoplasm	Polyribosomal RNA (>74S)*		Nonpolyribosomal RNA (≤74S)	
	Cpm in poly(A)	% total cpm in poly(A)	Cpm in poly(A)	% total cpm in poly(A)
None	1,700	3.2	1,720	0.35
+ EDTA	190	1.8	5,500	0.85

* See Fig. 3.

disrupted with EDTA. Similar results were obtained with cytoplasmic extracts treated with detergents to disrupt membranes. These data suggest an association of the poly(A) sequences with messenger RNA, since for this labeling period the predominant labeled polyribosomal RNA is mRNA, which is known to be displaced from the polyribosome sedimentation region to the sedimentation region of monosomes and sub-ribosomal particles by EDTA treatment (16).

To clarify this point, we incubated cells for only 30 min with $^{32}PO_4$, in order to label the mRNA before ribosomal RNA was significantly labeled. The ^{32}P distribution of the RNA isolated from these polyribosomes, as sedimented in a sucrose gradient, is shown in Fig. 4. This distribution of RNA species from 6 to 30S, with a maximum about 18S, is that commonly attributed to mRNA of HeLa cells (17).

The poly(A) content is shown in Fig. 4 as a percentage of the total RNA isolated from pooled fractions from four regions of the sucrose gradient. These quantities, which range from 2.5 to 5%, are in agreement with the poly(A) content of the total polyribosomal RNA reported in Table 2. The fact that the poly(A) content of polyribosomal RNA was not reduced by isolation with the hot phenol method and re-sedimentation through a sucrose gradient suggests a covalent association of small poly(A) sequences with the rapidly labeled mRNA. Denaturation–competition studies of the type carried out on the HnRNA are needed to establish this conclusion for mRNA.

Size of poly(A) sequences

A similarity in electrophoretic mobility of the poly(A) sequences derived from HnRNA species of different sedimentation velocities has already been noted. The mobilities on polyacrylamide gels of the poly(A) sequences from polyribosomal RNA and from total nuclear RNA are compared with some well-characterized marker RNA species in Fig. 5. Poly(A) of both origins is relatively homogeneous and displays similar mobilities. Lengths of 200 nucleotides would be estimated from plots of log molecular weight versus mobility constructed for the small well-characterized RNA markers (14). This can only be regarded as tentative in view of the lack of secondary structure in poly(A) at neutrality, as opposed to the marker RNA species. Composition analysis showed that AMP accounted for 99% of the ^{32}P in polyribosomal poly(A) and 91% in total nuclear poly(A). Since ribonucleases have been used to release the poly(A) from larger RNA molecules there may be nucleotides other than AMP at the 3′ ends, or infrequently within the poly(A) sequence.

The homogeneity of the total polyribosomal poly(A) in RNAs with widely differing sedimentation velocities (Fig. 4) again suggests that many mRNA molecules may contain a poly(A) sequence of uniform length. The inverse relationship of poly(A) content to the size of mRNA noted in Fig. 4 would be compatible with such a prediction. The most slowly sedimenting RNA fraction analyzed, with a poly(A) content of only 2.5%, probably contains a considerable proportion of labeled tRNA in addition to some mRNA.

Effect of actinomycin D on poly(A) synthesis

Actinomycin D concentrations (5 μg/ml) that reduce total RNA synthesis by 99% reduced poly(A) synthesis by 90%. That 10% of poly(A) synthesis escaped this inhibition may relate either to the well-known preferential effects of this drug on guanine-rich sequences in DNA or to an independent actinomycin-insensitive synthesis of poly(A) (1). It appears

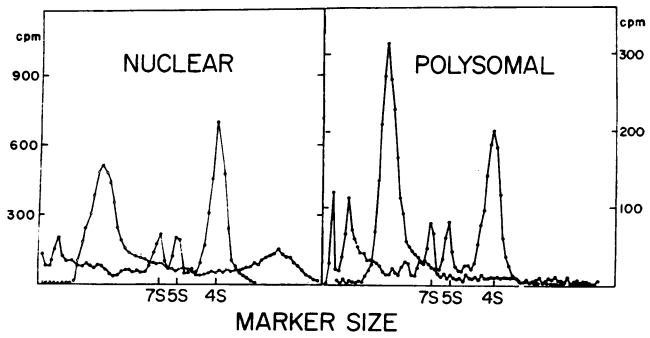

FIG. 5. Acrylamide gel electrophoresis of poly(A) isolated from labeled total nuclear RNA and rapidly-labeled polyribosomal RNA. Poly(A) was prepared from the two RNAs of cells labeled for 60 min with $^{32}PO_4$ as in the legends to Figs. 1 and 4. The poly(A) was electrophoresed together with [^{3}H]uridine-labeled HeLa cell cytoplasmic RNA that had been heated to separate 7S rRNA from the 28S rRNA. Electrophoresis, was for the equivalent of 17 hr at 35 V on 14-cm gels with 10% cross-linkage. The approximate seidmentation constants of the marker RNA species are indicated on the abscissa. O—O, poly(A); ●—●, marker mixture.

that about 90% of the poly(A) synthesized in HeLa cells is made in a transcriptive process.

DISCUSSION

Our experiments have defined the existence of poly(A) sequences within nuclear HnRNA and within rapidly-labeled polyribosomal RNA that is probably mRNA. For HnRNA our evidence strongly indicates covalent linkage of the poly(A) within the RNA sequence, while other considerations suggest that this may also be true of poly(A) associated with mRNA. Furthermore, inhibition of RNA synthesis with actinomycin D shows that most, if not all, of the poly(A) sequences are transcribed on a DNA template.

The size and relative abundance of the poly(A) sequences in both the HnRNA and mRNA fractions leads to a prediction that many of the RNA molecules within each class contain a poly(A) sequence. For HnRNA with $s > 45$ S, estimated molecular weight of $7\text{–}10 \times 10^6$ (5), a poly(A) sequence of 150–200 nucleotides would lead to a poly(A) content of 0.4–0.6%, in the range observed (Table 1) for the abundance of poly(A) in HnRNA. A similar calculation made for the total mRNA fraction, assuming an average molecular weight for that RNA of about 6×10^5 and a poly(A) sequence of 150–200 nucleotides per mRNA gives a predicted poly(A) content of 12–16%, considerably higher than the average figure we observed, about 4–5% (Fig. 4). The observed low value may mean either that not all mRNA molecules have a poly(A) sequence or that not all rapidly-labeled polyribosome RNA is mRNA (17). The result could also reflect a "nearest-neighbors effect" on poly(A) labeling due to sluggish labeling of the α-phosphate of ATP (22). This effect would be enhanced for mRNA, since the radioactivity accumulating in it during a pulse label of brief duration reflects RNA synthesized early in the labeling period (17), in contrast to HnRNA, which is turning over rapidly (13).

Poly(A) from both HnRNA and mRNA appears homogeneous in acrylamide gel electrophoresis, and the poly(A) of the two origins seems to have the same mobility relative to marker RNAs. Since mRNA is synthesized in the nucleus, the existence of similar poly(A) sequences raises the question of a

possible precursor–product relationship between HnRNA and mRNA. The poly(A) found in the rapidly-labeled RNA in polyribosomes almost certainly arises by transcription in the nucleus, and neither in our study nor in those of others (18) has a significant synthesis of small poly(A)-rich RNA been observed in HeLa cell nuclei. This suggests that the poly(A) sequences found in mRNA are in fact transcribed within larger RNA species, possibly HnRNA.

Previously it has been established that the HnRNA turns over very rapidly within HeLa nuclei; less than 10% of this RNA is likely to be transported to the cytoplasm, if any is (13). We observe approximately a 10-fold difference in poly(A) content between HnRNA and mRNA (Figs. 1 and 4). We therefore raise the possibility that each molecule of HnRNA transcribed contains within it one sequence of potential mRNA adjacent to a small poly(A) sequence. The HnRNA molecule would be rapidly degraded after or during transcription, and only a small portion of it including mRNA and poly(A) would be conserved and exported to the cytoplasm.

Evidence compatible with this model relating cytoplasmic mRNA and nuclear HnRNA has previously been obtained (8) in a study by DNA·RNA hybridization which revealed viral-specific sequences in both mRNA and HnRNA in SV40-transformed 3T3 cells. In several other studies evidence has been found relevant to the possibility that animal cell mRNA may be linked to poly(A) sequences. An adenylate-rich sequence associated with RNA from rabbit reticulocytes, which has the electrophoretic mobility attributed to globin mRNA, has recently been identified (19).* In another study it has been found that the electrophoretic mobility of putative globin mRNA is less than expected by an amount consistent with a length about two hundred nucleotides more than required on the basis of coding needs (20). Vaccinia virus-specific RNA polymerase has also recently been reported to synthesize poly(A) sequences in the course of mRNA synthesis, both *in vitro* and *in vivo* (21).

The postulated model raises two particular questions, the first of which is the function, if any, of the large portion of the HnRNA molecule that would be rapidly degraded soon after synthesis. However, a precedent exists in the processing of the 45S precursor to rRNA in human cells, where about 50% of each molecule is rapidly degraded soon after synthesis without serving any known function (5). The second question is what the function or functions of poly(A) sequences in HnRNA and mRNA might be. One possibility is that these sequences are recognition signals involved in the processing of HnRNA or the translation of mRNA or both.

In an independent investigation, Darnell, Wall, and Tushinski (personal communication; ref. 23) find that the rapidly hybridizing, highly reiterated sequences previously identified in their laboratory in HeLa mRNA are rich in adenylate, in agreement with our findings. By means of Me_2SO denaturation experiments they have concluded that the adenylate-rich sequences in mRNA are covalently bound.

Our present results are not sufficient to confirm the validity of the model for mRNA synthesis postulated above. In particular, we have not rigorously excluded the possibility of a minority of the HnRNA molecules being essentially tandem duplicate copies of poly(A) sequences joined by ribonuclease-sensitive sequences. It is also possible that poly(A) sequences are synthesized separately from HnRNA and are secondarily attached to the latter. Further studies relevant are in progress.

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* For evidence of its occurrence in mRNA of mouse sarcoma 180 ascites cells, see Lee, S. Y., J. Mendecki, and G. Brawerman, *Proc. Nat. Acad. Sci. USA*, **68**, 1331 (1971)(this issue). [Ed.]