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Properties of a small transcribed poly A sequence in heterogeneous nuclear RNA of HeLa cells

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ABSTRACT

A class of heterogeneous nuclear RNA (hnRNA) molecules contain an internal transcribed poly A sequence of close to 25 uninterrupted AMP residues. HnRNA molecules containing this sequence are separable from those containing the large 3' terminal poly A sequence on the basis of their differential affinity for oligo dT cellulose. The fact that the transcribed small poly A and the 3' terminal poly A are not found in the same hnRNA molecules even though both are present in similar size classes and that the small poly A is absent from cytoplasmic messenger RNA (mRNA) has led us to propose a scheme for mRNA processing in which the 3' end of the small poly A in hnRNA becomes a priming site for the post-transcriptional addition of the large poly A.

INTRODUCTION

Poly A preparations from ribonuclease digests of hnRNA of HeLa cells purified on oligo dT cellulose contain a small AMP-rich component which readily separates from the large poly A sequence during electrophoresis. While investigating the location of the large poly A in periodate oxidized nuclear RNA molecules which had been reduced with labeled sodium borohydride, it was noted that in contrast to large poly A, this rapidly migrating species did not become labeled (1). It was concluded that unlike large poly A, this small AMP-rich species was not at the 3' end of hnRNA molecules.

A comparative study of the effects of controlled doses of actinomycin D and 3' deoxyadenosine on the biosynthesis of the two sequences clearly showed that different mechanisms were involved (2). Synthesis of the large poly A was, as expected, inhibited markedly by 3' deoxyadenosine, while small poly A was not. Conditions of actinomycin treatment were also found which could abolish small poly A synthesis without greatly reducing large poly A synthesis (2). It was apparent that the small poly A was a transcribed sequence not derived from the large poly A nor was it a special size class of 3' terminal poly A.

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We now report some of the properties of this sequence and its distribution in RNA molecules in the nucleus and cytoplasm of HeLa cells. From these data we speculate on a possible role for this smaller transcribed poly A sequence in the synthesis of the large poly A at the 3' ends of hnRNA.

MATERIALS AND METHODS

Sources for most of the materials used have been reported previously (3). E. coli alkaline phosphatase (BAPF) and snake venom phosphodiesterase were from Worthington Biochemical Corp. The latter was extensively purified by gel filtration on Sephadex G100 and ion exchange chromatography on DEAE cellulose (Nakazato, unpublished experiments).

Cell Culture, Labeling, Fractionation and RNA Extraction. These methods have been described for HeLa cells in another publication (4).

Separation of Poly A (+) from Poly A (-) hnRNA. The use of oligo dT cellulose for effecting this separation has been previously described (3,4).

Isolation of Large and Small Poly A Sequences. Poly A sequences were isolated by a modification (2) of an earlier procedure (5) that permits a quantitative binding of small poly A sequences to oligo dT cellulose. Electrophoresis of poly A in 10% aqueous gels (2,5) and in 98% formamide gels have been described (6).

Enzymatic Treatments.

Alkaline phosphatase. Oligo A recovered as an ethanol precipitate was washed with diethyl ether and dried in air. The powder was re-dissolved in 1.0 ml of 0.02M Tris:HCl pH 8.5 containing 10 mM EDTA before E. coli alkaline phosphatase was added at a concentration of 0.5 unit per A_{260} absorbance unit of RNA. After 30 minutes at 37°, the reaction was chilled to 0°, NaCl and SDS were added to final concentrations of 0.5M and 0.5% respectively. The sample was then passed over a 0.5 x 0.5 cm column of oligo dT cellulose at 25°C and poly A was eluted with ETS containing 0.5% SDS. The eluted oligo A was extracted three times for three minutes with equal volumes of phenol at 60°. The oligo A was precipitated from the pooled aqueous extracts with ethanol after the addition of 5 A_{260} units of carried RNA (previously treated with alkaline phosphatase) and NaCl to a final concentration of 0.1M.

Phosphodiesterase. Twenty µl of purified snake venom phosphodiesterase (0.5 mg/ml in 0.02M Tris·HCl, pH 8.0) was added to the phosphatased oligo A in 0.005 ml of 0.25 M Tris·HCl, pH 8.5 containing 0.5 µl of chloroform to prevent bacterial growth. After six hours at 37° an aliquot was removed for nucleotide analysis for the experiment of Fig. 7 (left panel). To the remain-

ing digest another 20 μl of phosphodiesterase was added along with 5 μl of 0.5M Tris·HCl, pH 8.5. Digestion was continued for another 12 hours (Fig. 7, right panel).

The 5' nucleotides of A, C, G and U were added as markers to both the 6 and 18 hour aliquots before 5' nucleotides were separated by high voltage paper electrophoresis (7).

Ribonuclease. Use of RNase T_1 and RNase A for the isolation of poly A and oligo A have been described (2,5). Modifications are cited in legends to Figures or Tables.

RESULTS

Localization of Oligo A in RNA of the Nucleus and Cytoplasm. We have developed conditions which allow an essentially quantitative binding of denatured poly A containing hnRNA molecules to oligo dT cellulose without significant degradation (4) and Fig. 1B. A brief heat treatment at 63° in 70% DMSO has relatively small effects on the sedimentation properties of total

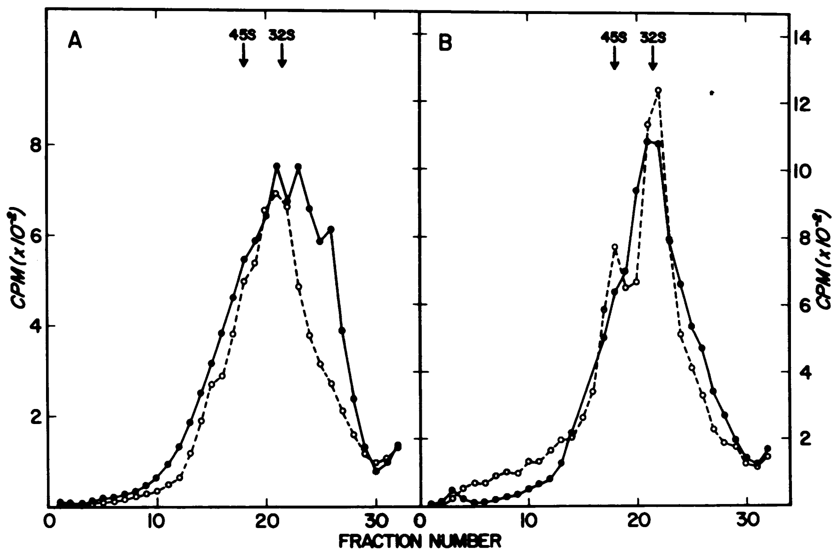

Fig. 1. Sedimentation profile of nuclear pA (+) and poly A (-) RNA. Nuclear RNA of HeLa cells labeled for 4 hrs with ^{32}P was separated into poly A (+) and poly A (-) RNA and centrifuged through 15% to 30% linear sucrose density gradient in NETS in a Spinco SW40 rotor for 13 hrs at 18,800 rpm after treatment with DMSO as described (3). Total nuclear RNA with and without DMSO treatment was also sedimented. A, poly A (+) nuclear RNA —●—●— and poly A (-) nuclear RNA —O—O—; B, total nuclear RNA with (—●—●—) and without (—O—O—) DMSO treatment.

nuclear RNA of HeLa cells labeled for 4 hours with ^{32}P (Fig. 1B). A similar treatment has been shown to be sufficient to denature the hnRNA of HeLa cells. The sedimentation profile of such RNA is essentially the same in aqueous and 99% dimethylsulfoxide (DMSO) sucrose gradients (8). Subsequent binding of this RNA to oligo dT cellulose has little effect on the sedimentation properties of either bound or unbound fractions (Fig. 1A). Figure 2A shows the large and small poly A sequences recovered from the ribonuclease digests of the unfractionated total nuclear RNA. Fig. 2B shows that oligo dT cellulose has produced nearly a complete separation of the large and small poly A se-

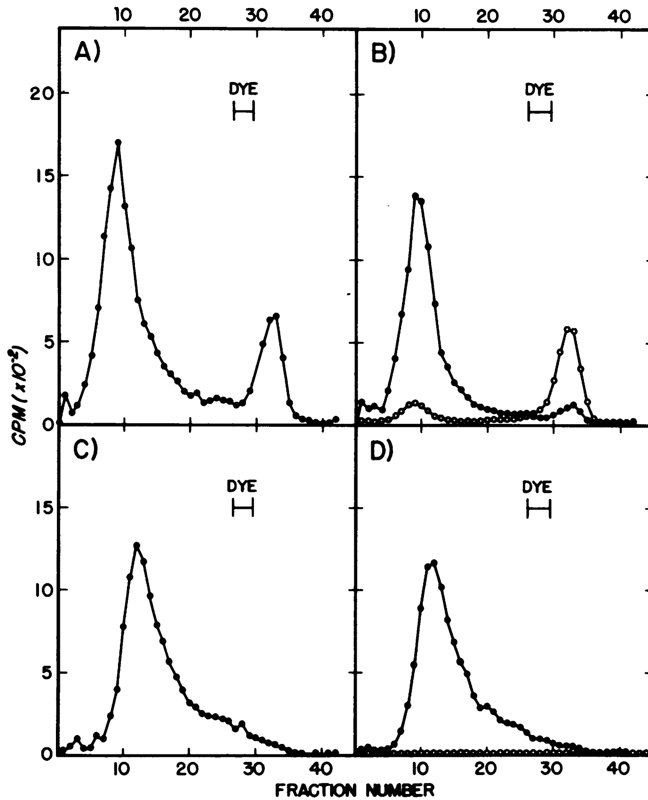

Fig. 2. Distribution of large and small poly A in cytoplasmic and nuclear RNA of HeLa cells. 4 hr labeled ^{32}P cytoplasmic and nuclear RNA of HeLa were separated into poly A (+) and poly A (-) fractions (3,4). Poly A's isolated from each fraction after nuclease treatment were further purified by a second binding and elution from oligo dT cellulose as described in the legend to Fig. 3. Poly A's from A) total nuc. RNA; B) poly A (+) nuclear RNA (●—●) and poly A (-) nuc. RNA (O—O); C) total cyt RNA and D) poly A (+) cyt RNA (●—●) and poly A (-) cyt RNA (O—O), were electrophoresed in 10% polyacrylamide gels (—):BPB dye marker.

quences. Since there is little evidence for degradation of the RNA during these manipulations (Fig. 1A), we conclude that the small transcribed poly A's are primarily in hnRNA molecules which do not contain the large poly A sequence. As is the case for poly A, they are found in all size classes of hnRNA with 30% sedimenting faster than 45S after short labeling periods (2). This distribution, plus the insensitivity of oligo A (2), but not ribosomal RNA synthesis (10) to inhibition by 3' deoxyadenosine, would eliminate preribosomal RNA as a major source of oligo A sequences.

Figures 2C and 2D show little evidence for a small poly A sequence of this size in the cytoplasm after 4 hours of labeling. This had been noted earlier in short labeling times as well (2). The diffuse spread of rapidly migrating radioactivity on these gels is primarily composed of AMP-rich sequences arising from the shortening of poly A during this relatively long labeling period (9).

Purification of Oligo A. The small poly A sequence is usually contaminated with RNA fragments not rich in AMP that are most easily removed by a second binding to oligo dT cellulose as seen in Figure 3. The fraction re-

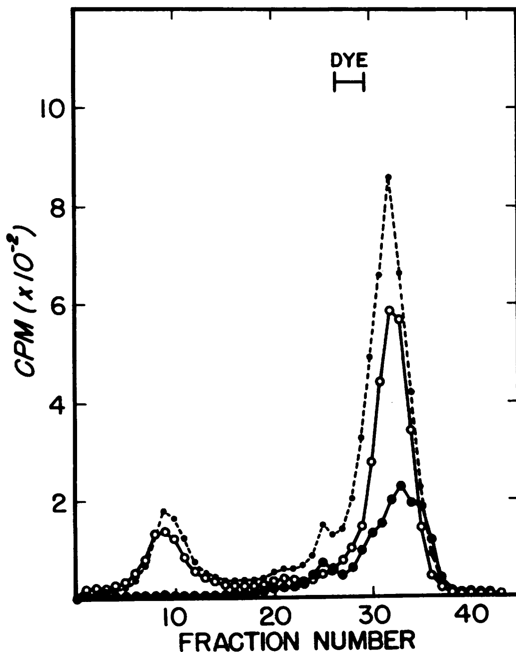

Fig. 3. Purification of small poly A by a second oligo dT binding. Poly A fraction (●—●) isolated from nuclear poly A (-) RNA after nucleasing by binding and eluting from oligo dT cellulose was rebound to oligo dT cellulose at 23° in 0.5M NaCl in 0.2% SDS, 0.01M Tris pH 7.0, 0.01M EDTA and separated into bound (O—O, 68.8% of "poly A fraction") and unbound (●—●, 31.2%) fractions described in Fig. 2. Electropherograms run in parallel are plotted in one figure. (—):dye marker. Base compositions of sample –O–O– was 2.3% C, 89.5% A, 3.9% G and 4.3% U; –●–●– was 32.2% C, 9.8% A; 44.0% G and 14% U.

tained by oligo dT cellulose is now considerably more homogeneous (Fig. 4A) and is highly enriched in AMP as the data of Table 1, experiment 1 shows. Fig. 4B shows that the sequence is not detectably shortened by a second treatment with a 50-fold increase in the RNase A concentration, suggesting it is uninterrupted by pyrimidine nucleotides.

The Size and Composition of Oligo A. The average length of oligo A sequences had been estimated previously to be in the 20-30 nucleotide range from comparisons of electrophoretic mobilities of other natural poly A sequences found at 3' termini from which an independent estimate of length was obtained by end groups analysis (4). However, the possibility of a non-linear relationship between electrophoretic mobility and length for short oligo A's in these gels created a need for more definitive measurements. The electrophoretic mobility of oligo A was compared with both smaller and larger oligo A markers of known length in both aqueous and denaturing gels (Fig. 5). Oligo A migra-

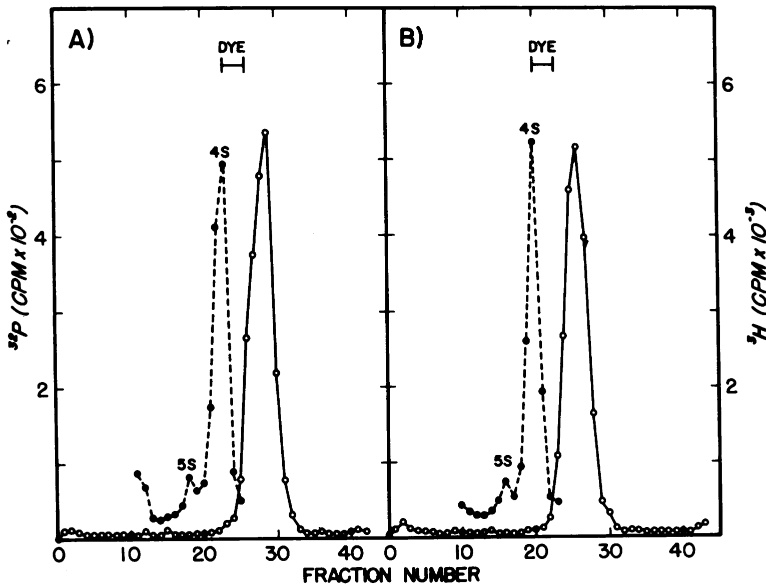

Fig. 4. Inability of RNase A to hydrolyze purified oligo A. Oligo A isolated from nuclear poly A (-) RNA was recovered from a 10% polyacrylamide gel after electrophoresis and was purified by binding and eluting from oligo dT cellulose as described in Fig. 3. Two aliquots were incubated for 30 min at 37°C with and without 4 μg of RNase A in 1.2 ml of 0.025M Tris HCl pH 7.4 containing 0.2M NaCl and 1 O.D. units of yeast soluble RNA. After adding SDS to 0.7%, 40 μl aliquots of each reaction were electrophoresed on 10% polyacrylamide gel with ^{3}H adenosine labeled HeLa cytoplasmic RNA as a marker. A) without RNase; B) with RNase. O—O: small poly A, ●—●: ^{3}H cyt RNA, (—): dye marker.

TABLE 1

COMPOSITION AND SIZE OF A TRANSCRIBED POLY A SEQUENCE

Exp	Treatments 1	2	3	Nucleotide Composition C*	A	G	U	Total ^{32}P Analyzed
					%			CPM
1a	RNase A + T_1	---	KOH	0.7	93.9	3.8	1.5	7,180
	RNase A + T_1	phosphatase	KOH	0.1	99.0	0.2	0.7	17,400
1b	RNase A + T_1	---	KOH	0.6	93.9	3.5	2.0	11,500
	RNase A + T_1	phosphatase	KOH	0.0	98.1	0.0	1.9	12,100
2a	RNase A + T_1	phosphatase	phosphodiesterase	1.3	93.5	3.8	1.3	40,400
2b	RNase A + T_1	phosphatase	phosphodiesterase	1.2	92.9	4.3	1.6	23,500
3a	RNase A	---	KOH	1.2	87.0	8.0	3.8	21,800
	RNase A	phosphatase	KOH	0.5	88.7	10.8	1.1	25,000
3b	RNase A	---	KOH	1.5	85.4	9.5	3.6	14,000
	RNase A	phosphatase**	KOH	0.1	88.4	10.1	0.6	15,500
4a	RNase A	phosphatase	phosphodiesterase	1.7	84.8	9.9	3.6	16,000
4a'	RNase A	phosphatase	phosphodiesterase	1.8	84.6	10.1	3.6	23,500
5a	RNase T_1	---	KOH	2.6	89.0	3.6	4.2	9,630
5a'	RNase T_1	---	KOH	2.8	88.0	3.6	4.6	5,390

* Corrected for a contaminant derived from alkaline hydrolysates of poly A which co-migrates with CMP (14).

**The oligo A sequence recovered from the RNase A digest was treated with alkaline phosphatase a second time before alkaline hydrolysis.

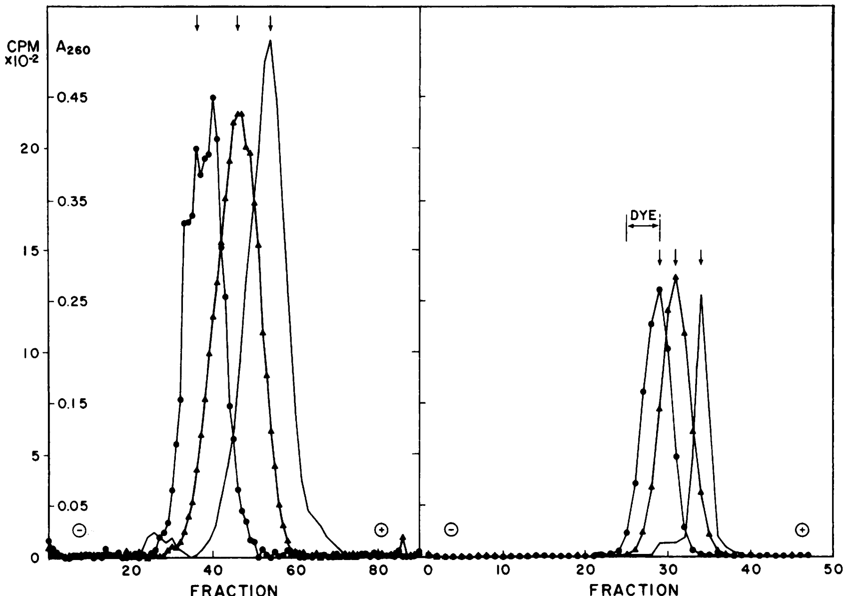

Fig. 5. Comparative electrophoretic mobilities of oligo A's in aqueous and denaturing polyacrylamide gels. HeLa nuclear ^{32}P-oligo A (-Δ-) purified as described in Fig. 4 was treated as before with RNase A and RNase T_1 before binding to oligo dT cellulose. The eluted oligo A was then treated with E. coli alkaline phosphatase and recovered on oligo dT cellulose. This ^{32}P-oligo A was mixed with an ^{3}H-oligo $\bar{A}$ fraction (-O-) that moved somewhat slower during electrophoresis than HeLa oligo A. It has been prepared from an RNase T_2 and E. coli alkaline phosphatase digest of ^{3}H-poly A (Miles Laboratories). An average chain length of 45 was determined from the ^{3}H in AMP and adenosine in a total RNase T_2 digest of this oligo A from eluates of gel slices 29 through 41 of the denaturing gel shown in the left panel (above). These two components plus 50 µg of a $p(A)_9A$ (—) (from Collaborative Research, Waltham, MA) were co-electrophoresed either in a 10 cm 10% aqueous (right panel) or a 15% denaturing gel in 98% formamide (left panel). Each gel was scanned for absorbance at 260 nm in a Gilford linear transport system before cutting into 1 mm slices with an electric slicer (Hoefer Scientific). Each slice was eluted and counted to obtain the data on left panel while 2 adjacent slices were combined for each fraction in right panel.

ted slower than A_{10}, but faster than A_{45} in both aqueous and 98% formamide gels suggesting that the oligo A sequences did, in fact, fall within a length range of 20-30 nucleotides as suggested. Although the 3 components were clearly separated according to size in both gel systems, overlap is evident, suggesting diffusion in these gels is limiting resolution. Anion exchange chromatography, however, could completely separate the A_{10} marker from the larger components and provides clear evidence that oligomers less than 15 nucleotides are not present in oligo A. Resolution of oligomers larger than 15 is generally not feasible on DEAE cellulose but the oligo A from hnRNA eluted well ahead of the A_{45} marker on this column although overlap remains. (Fig. 6)

An independent estimate of average oligo A length can also be made from the composition of this oligo A, since it appears that 70-80% of these sequences have a GMP at the 3' side (experiments 1 and 2, Table 1). GMP is the only nucleotide present in sufficient quantity to be present once in a sequence of 25 nucleotides (i.e., about 4%). More importantly all ^{32}P can be removed from GMP with phosphomonesterase establishing its 3' location and also indicating that these oligo A's are uninterrupted since phosphatased preparations are 99% AMP. It is worth noting that the composition of the oligo A

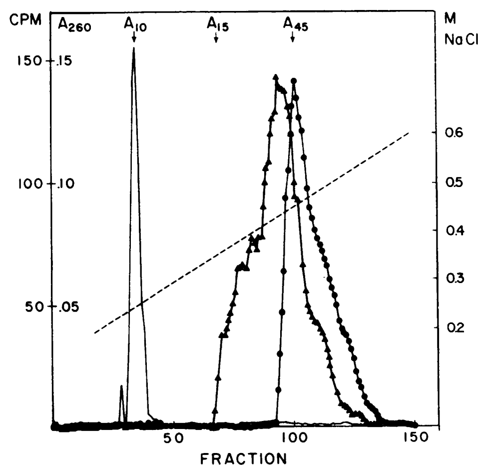

Fig. 6. Anion exchange chromatography of the oligo A sequences in the hnRNA of HeLa cells. A mixture of the 3 components described in Figure 5 were co-chromatographed on a 30 x 0.5 cm DEAE Sephacel column equilibrated with 10 mM $NaPO_4$, pH 7.0, 5 mM EDTA and 7M urea. The components were eluted in this buffer with 200 ml of a linear NaCl gradient beginning at 0.2M and ending at 0.6M. ^{32}P-oligo A of hnRNA (-Δ-), ^{3}H-oligo A (-O-), $p(A)_9A$ (—); NaCl concentration (----). The arrow at A_{15} marks the midpoint of the elution volume containing $p(A)_{14}A$ in an identical column.

fraction obtained from 5' nucleotides recovered from a snake venom phosphodiesterase digestion of phosphatased oligo A prepared from a different hnRNA sample by the treatment used in experiments 1 and 2 had the identical composition (Table 1).

Composition of Larger Oligo A Fragments. This relative homogeneity of the 3' ends encouraged us to analyze more limited nuclease digests that might leave additional nucleotides at 3' and 5' ends of oligo A. A fragment released by RNase A digestion was purified as described for Fig. 3. Experiment 3, Table 1 shows that majority of these sequences contained approximately 2 GMPs and 1 UMP for every 25 AMP residues. In this preparation most of the UMP was at the 3' end since, in contrast to GMP it nearly disappeared from the alkaline hydrolysates of phosphatased preparations, but not from phosphatased preparations hydrolyzed with snake venom phosphodiesterase where the 3' terminal nucleotide is recovered as 5' UMP (experiment 4). A minority of these sequences must have CMP at the 3' end since a rather low initial radioactivity in CMP was removed from alkaline hydrolysates of the phosphatased sample (experiment 3), but not from phosphodiesterase hydrolysates (experiment 4).

Two observations derived from 3' exonuclease treatment of this RNase A product with snake venom phosphodiesterase allow us to assign the extra GMP in this sequence to the 3' side of oligo A. First, had this extra GMP been at the 5' end of the phosphatased oligo A sequence, it would have been released as guanosine and not 5' GMP as found (Table 1). Additional evidence for a second GMP at the 3' end of the sequence is found in the timed release of UMP and GMP during phosphodiesterase digestion. Fig. 7 shows that almost all of the UMP and 75% of the GMP are released before even half of the AMP is released. Although the data in Fig. 7 actually indicates close to 50% of the AMP has been released, this is certainly an over-estimate since no correction has been made for the ^{32}P present as undigested oligonucleotides that overlap the AMP region (see Fig. 7). Such contamination is not found in the 5' GMP and 5' UMP regions. Although the presence of AMP residues within the -GGU- sequence is not ruled out, it appears that many of the oligo A sequences in hnRNA may have -GGU- at the 3' end.

The composition of a sequence purified from a RNase T_1 digest is reported in experiment 5 of Table 1. As expected it contains one GMP per 25 AMP residues. Slightly more than one UMP, but less than one CMP were found for each GMP residue, suggesting that sequences at the 5' ends of oligo A may be heterogeneous, but that approximately 2 pyrimidine residues separate the 5' end of oligo A from a GMP in the direction of the 5' end of hnRNA.

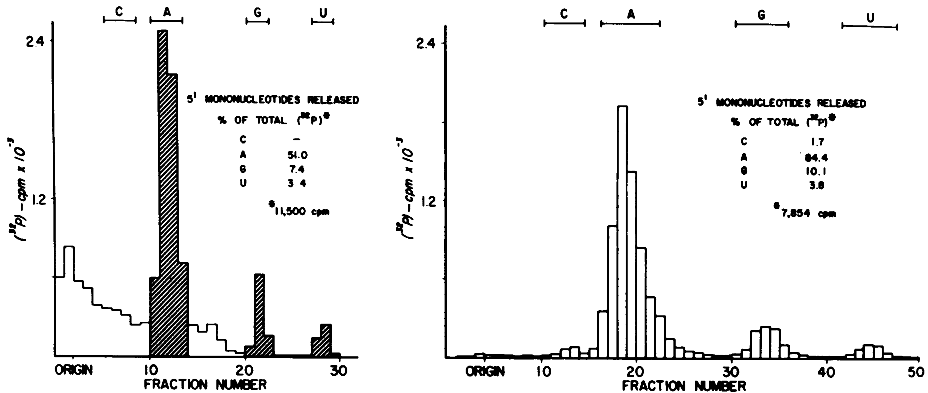

Fig. 7. Release of 5' nucleotides from an oligo A sequence purified from a RNase A digest of poly A (-) hnRNA. The oligo A sequence recovered on oligo dT cellulose from a RNase A digest of the poly A (-) hnRNA fraction (3,4) was purified as described in Fig. 3 and was treated sequentially with E. coli alkaline phosphatase and snake venom phosphodiesterase as described in Methods. Left panel is electropherogram of 5' nucleotides recovered after digestion for 6 hours at 37°C. Right panel after an additional 12 hours as described in Methods.

DISCUSSION

The observations reported here on the distribution of oligo A and poly A sequences in HeLa cell mRNA (Fig. 2) and the relatively low sequence complexity of the 3 nucleotides adjacent to the 3' end of oligo A have led us to consider a role for oligo A sequences as processing sites that may serve as priming sites for poly A synthesis, an idea once suggested by Scherrer (11).

The fact that oligo A and poly A sequences are not present in the same hnRNA molecules of any size class and that mRNA appears to lack oligo A could be accounted for by the series of steps outlined in Fig. 8. According to this scheme oligo A occurring at some unspecified site within hnRNA undergoes cleavage at or near its 3' end to produce a 3' hydroxyl group on the terminal AMP that subsequently becomes the site for the polymerization of new AMP residues by poly A polymerase. Sequences released by the endonuclease, as well as others from the 5' ends of hnRNA which are not destined for export to cytoplasm as mRNA are then degraded. After a series of post-transcriptional modifications the polyadenylated RNA is transported to the cytoplasm as mRNA.

This model leads to two predictions that might be tested experimentally. One is that a small piece at the 5' end of the mature poly A sequence arises by transcription rather than by post-transcriptional polyadenylation. A com-

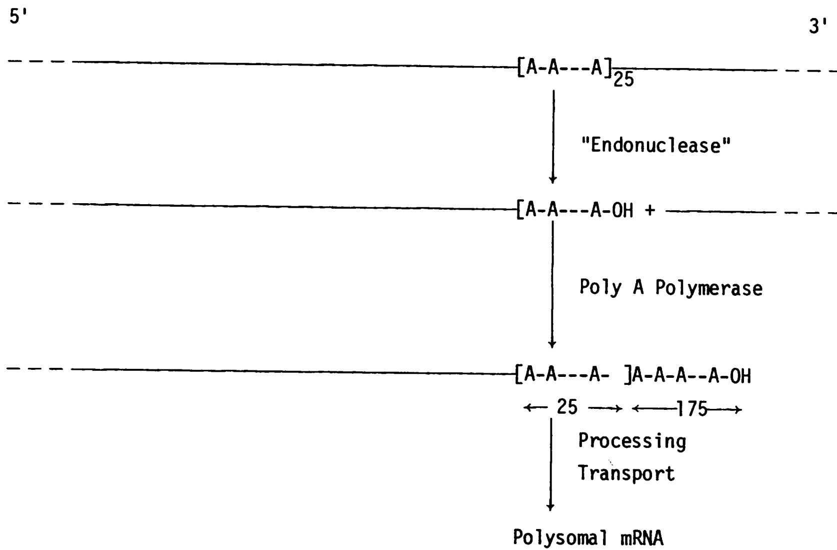

Fig. 8. A model providing a role for a transcribed short poly A sequence as a priming site for poly A synthesis.

bination of *in vivo* and *in vitro* transcription systems in which poly A synthesis can be manipulated may provide the appropriately labeled poly A sequences to answer this question (12). A second prediction is that the 5' side of the oligo A sequence in hnRNA should contain the nucleotide sequences found in the 3' terminal region adjacent to the poly A sequence of hnRNA and mRNA. With respect to the latter it can be said that the very limited data available for nucleotides at the 5' end of oligo A purified from a RNase T_1 digest are not incompatible with those published for the nucleotides attached to the 5' end of the poly A sequence of HeLa mRNA (13,14). This could be examined more rigorously with cDNA probes for specific mRNA molecules, since it is unlikely that mixed RNA populations will have sufficient sequence homology in regions surrounding either poly A or oligo A to answer this question.

The model also predicts that an endonuclease recognizes a specific region at the 3' side of oligo A where processing begins prior to polyadenylation. The limited structural information on sequences surrounding oligo A plus the general lack of knowledge of the nucleases involved in RNA processing in eukaryotes makes this a difficult point to pursue experimentally.

The so-called "splicing reactions" recently reported for animal viruses that link pieces of viral mRNA that are transcribed from non-continuous regions of the viral genome (15,16,17) are likely to be involved in the proces-

sing of cellular RNA as has been shown for adenovirus hnRNA (18). The model of Figure 8 would be compatible with data recently published for the sequential processing of adenovirus nuclear transcripts into late messenger RNAs, where poly A addition precedes splicing and in fact, probably occurs even before completion of the large nuclear adenovirus transcript (18).

Even though the observed distribution of oligo A sequences may merely point to the existence of distinct, perhaps metabolically unrelated types of RNA, the fact that two classes of hnRNA in HeLa cells that encompass a similar range of sizes can be separated on the basis of a known sequence difference may open up new possibilities for understanding the function of hnRNA. It is of interest that the oligo U sequences in HeLa cell RNA that are similar to oligo A in length do not show this restricted distribution, but are found in both poly A containing and poly A lacking hnRNA and in a fraction of mRNA as well (3). Developing sea urchin embryos also contain these two classes of hnRNA, as well as a third more abundant class that contains neither poly A nor oligo A (19). The relative amounts of each of these classes differ as a function of development of the embryo (20).

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