Proc. Nat. Acad. Sci. USA
Vol. 71, No. 1, pp. 200–204, January 1974

Differential Metabolism of Large and Small Poly(A) Sequences in the Heterogeneous Nuclear RNA of HeLa Cells

(transcriptional synthesis/messenger RNA/3′-deoxyadenosine/actinomycin D)

HIROSHI NAKAZATO, MARY EDMONDS, AND DAVID W. KOPP

Department of Biochemistry, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Communicated by Norman Davidson, September 10, 1973

ABSTRACT **The heterogeneous RNA of the HeLa cell nucleus contains short internal poly(A) sequences which, in contrast to the longer poly(A) sequences at the 3′ ends, are not found in messenger RNA of the cytoplasm. A distinct origin for each of these homologous sequences is evident when the effects of actinomycin D and 3′-deoxyadenosine on their biosynthesis are compared. The shorter poly(A) appears to be transcribed, while the longer one does not. A kinetic analysis of the metabolism of each type of poly(A) sequence within different size classes of HnRNA after treatment of the cells with 3′-deoxyadenosine reveals distinctive distribution and metabolism of these sequences and also provides insight into mechanisms proposed for the generation of messenger RNA from these large RNA molecules in the nucleus.**

We have recently detected a short poly(A) sequence in the heterogeneous nuclear RNA (HnRNA) of HeLa cells (1) which, in contrast to the large poly(A) sequence described earlier (2), is not found in the cytoplasm nor at the 3′-end of HnRNA (1). The existence of two classes of poly(A) sequences in HnRNA, only one of which is at the 3′-terminus, which are readily separated on the basis of length presented an experimental opportunity for comparing the mechanisms of their biosynthesis.

We wish to report the results of two types of experiments which clearly reveal distinct mechanisms for the biosynthesis of the two poly(A) species. First, comparison of the differential effects of actinomycin D and 3′-deoxyadenosine on the biosynthesis of the two sequences clearly points to a transcriptional synthesis of small poly(A) which is not evident for the larger poly(A) sequence. In a second set of experiments, the distribution of large and small poly(A) sequences in different size classes of HnRNA has been examined. Changes in the distribution of label in the two sequences within each size class after the addition of 3′-deoxyadenosine again emphasizes the unique metabolism of each type of sequence.

The relevance of these data to models currently proposed to account for the generation of messenger RNA by mechanisms involving processing of large HnRNA molecules will be discussed.

MATERIALS AND METHODS

Cell Culture and Labeling. Suspension cultures of HeLa cells were maintained at concentrations of 2 to 5 × 10^5 cells per ml as previously described (2). Cells were concentrated to 4 × 10^6 cells per ml for labeling with [*2,8*-^{3}H]adenosine in Eagles' minimal medium supplemented with 5% normal calf serum.

Cell Fractionation. Cells were ruptured in a Dounce homogenizer after hypotonic swelling as previously described (2). Nuclei recovered by centrifugation were rinsed once with 1 mM NaCl: 10 mM Tris pH 7.4: 1.5 mM $MgCl_2$ but not with detergents. Cytoplasm to be used for total cytoplasmic RNA isolations was first freed of mitochrondria by centrifugation at 13,000 × *g* for 10 min. All RNA was extracted by the hot phenol-sodium dodecyl sulfate method as described earlier (2).

Poly(A) Isolation. The poly(A) isolation procedure differed somewhat from that described previously (2) when it was found that not all of the small poly(A) fraction remained bound to oligodeoxythymidylate cellulose filters during the 0.1 M NaCl:0.01 M Tris (pH 7.4) wash at 23°. Washing was, therefore, done at 0° with ten 3-ml portions of this solution. The temperature of the filter was then raised to 23° before the poly(A) fraction was eluted with seven 1-ml washes of 0.01 M Tris (pH 7.4).

Gel Electrophoresis of Poly(A). Poly(A) fractions released from oligo(dT)-cellulose were precipitated with ethanol and prepared for electrophoresis on 11.5 cm of 10% polyacrylamide gels, as described previously (2). After electrophoresis at 60 V, gels were transferred to a Maizel fractionator, where the bottom of the gel was protected with a small piece of dyed gel which also marked the end of the gel. Gels were smashed into 40–45 equal fractions, each of which was suspended in about 1.8 ml of water in scintillation vials. The fractions were shaken overnight, after which 10 ml of toluene omnifluor (New England Nuclear Corp.) containing 20% Triton X-100 was added and the radioactivity was measured.

RESULTS

Effect of Actinomycin D and 3′ Deoxyadenosine on Poly(A) Synthesis. The data of Table 1 and Fig. 1 clearly show that the small poly(A) sequence is not generated from the larger one either *in situ* or during the purification and processing of poly(A) sequences. These conclusions are based primarily on the different effects the two drugs have on the synthesis of each poly(A) sequence, as well as on the fact that the small poly(A) sequence is not found in cytoplasmic poly(A) (Fig. 1*C*).

The marked reduction in synthesis of the small poly(A) in actinomycin D, which essentially parallels the synthesis of the bulk of the nuclear RNA, indicates a transcriptive mechanism for the biosynthesis of this sequence, while its relative lack of effect on the synthesis of large poly(A) sequences supports the conclusions of Darnell, *et al.* that the large poly(A) sequence is added after the transcription of HnRNA (3).

Similar differences, but in the opposite direction, are found when poly(A) synthesis is measured in the presence of 3′-deoxyadenosine, an adenosine analogue which has relatively

Abbreviations: HnRNA, heterogeneous nuclear RNA; SV40, simian virus-40.

TABLE 1. *Effect of actinomycin D and 3'-deoxyadenosine on the synthesis of poly(A) sequences**

Treatment	Nuclear			Cytoplasmic		
	RNA	Large poly(A)	Small poly(A)	RNA	Large poly(A)	Small poly(A)
	cpm × 10^{-3}					
Untreated	43,800	560	187	5,150	287	Trace
+ 3'-Deoxyadenosine	30,700	42.1	166	2,590	38.7	Trace
+ Actinomycin D	2,130	252	29.3	1,070	31.9	Trace

* All poly(A) fractions are from gels described in Fig. 1. Values of ^3H were obtained by combining the radioactivity found in the nine fractions of the large and the 13 fractions of the small poly(A) which were most highly labeled. (The numbers shown in the tables have been multiplied by 10^{-3}.)

little effect on HnRNA synthesis but which markedly inhibits the synthesis of 45S pre-ribosomal RNA (4). The inhibition effect undoubtedly accounts for the 30% reduction of total nuclear RNA synthesis seen in Table 1. The lack of inhibition of synthesis of the small poly(A) sequence seen here again parallels the lack of effect of this drug on HnRNA synthesis; however, in the same cells the synthesis of large poly(A) is essentially abolished. The marked decrease in labeled poly(A) sequences in the cytoplasm seen in Fig. 1 would also be expected from the reported effects of 3'-deoxyadenosine on the synthesis of messenger RNA (3, 5).

Although actinomycin D had a relatively small effect on the synthesis of the large nuclear poly(A) in this experiment, the transfer of the large poly(A) sequences to the cytoplasm as mRNA is apparently inhibited, since at these high levels (5 μg/ml) very little of the large poly(A) appeared in the cytoplasm. Although the size of HnRNA molecules containing large poly(A) sequences was not examined in this experiment, the failure to observe transport of mRNA to the cytoplasm suggests that processes other than transcription itself may have been altered in these cells. It has been reported that in HeLa cells even low doses of actinomycin D (0.04 μg/ml) can suppress the appearance of mRNA in polyribosomes (6).

Distribution of Large and Small Poly(A) Sequences in HnRNA after Addition of 3'-Deoxyadenosine. It has already been shown that the large poly(A) sequences are distributed within all size classes of HnRNA (1, 7), and inspection of Fig. 3 shows this to be true for the small poly(A) sequences as well. It is apparent, however, that both the relative and absolute amounts of each type of poly(A) sequence in different size classes of HnRNA are not the same. The smaller HnRNA molecules have a relatively higher proportion of short poly(A) sequences, since the ratio of large to small poly(A) is 2.1 in fraction III *versus* 4.8 in fraction I. The small HnRNA molecules also contain far fewer large poly(A) sequences which, in this experiment, meant that about 83% of the poly(A)-containing HnRNA molecules sedimented more rapidly than 32S RNA (i.e., fractions I and II).

If a pool of mRNA of sizes found on polysomes exists in the nucleus, it would be found in fraction III. Since fraction III contains only 17% of the large poly(A) sequences, the steady state pool of poly(A)-containing mRNA would appear to be much smaller than the pools of the poly(A)-containing RNA in fractions I and II. That a steady state has apparently been achieved in this experiment is indicated by the fact that labeling of nuclear RNA does not increase in the control samples during the 45-min chase (Table 2).

Since 3'-deoxyadenosine abolished the synthesis of the large poly(A) (Fig. 1), it was possible to study the fate of pre-labeled large poly(A) and to compare it with that of small poly(A) in these different size classes of HnRNA.

The effects of a 45-min 3'-deoxyadenosine chase on total nuclear RNA seen in Fig. 2 are consistent with previously reported effects of this drug on nuclear RNA synthesis (4, 5). The disappearance of label from the 45S region of the gradient is expected, since synthesis of 45S RNA is inhibited. Processing of pre-labeled 45S RNA to 32S and 28S RNA, however, con-

TABLE 2. *Distribution of large and small poly(A) sequences in HnRNA after addition of 3'-deoxyadenosine*

Min after addition of 3'-deoxyadenosine	Total nuclear RNA	Large and small poly(A) in different sizes of HnRNA*					
		I		II		III	
		Large	Small	Large	Small	Large	Small
	cpm × 10^{-6}†	cpm × 10^{-3}‡					
+ 3'-Deoxyadenosine							
15	19.7	55.7	9.08	55.5	20.8	17.2	10.4
30	18.5	29.5	12.3	42.0	21.0	13.8	8.66
45	17.3	25.6	10.8	25.4	21.9	5.35	9.84
Control							
15	22.1	—	—	—	—	—	—
30	22.6	—	—	—	—	—	—
45	22.9	59.0	12.3	60.0	18.9	23.8	11.2

* All poly(A) fractions are from gels described in Fig. 3. Value for ^3H were obtained as described for Table 1.
† The numbers shown have been multiplied by 10^{-6}.
‡ The numbers shown here have been multiplied by 10^{-3}.

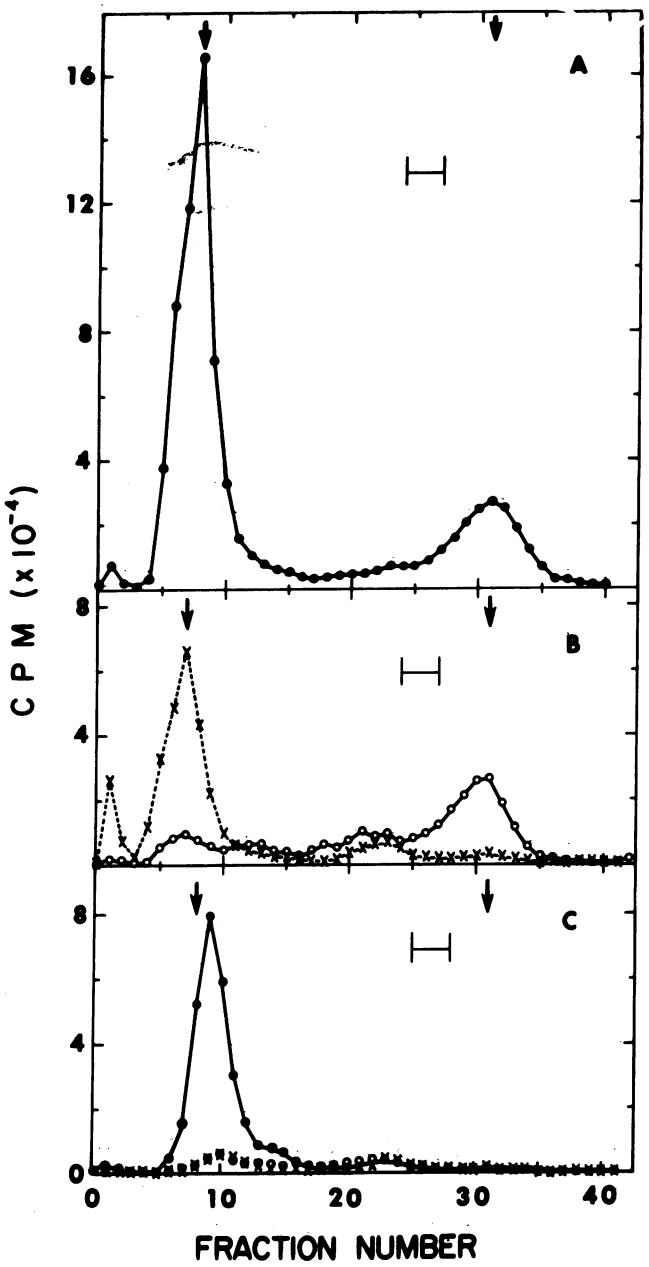

FIG. 1. Polyacrylamide gel electrophoresis of poly(A) isolated from nuclear and cytoplasmic RNA treated with actinomycin D or 3′-deoxyadenosine. Cells (12×10^7) were suspended in 30 ml of culture medium, incubated for 25 min, and then divided into three equal portions. Actinomycin D (10 μg/ml) was added to one portion and 3′-deoxyadenosine (50 μg/ml) was added to one portion. The third portion received no drugs and served as a control. After 2 min, 0.5 mCi of [^{3}H]adenosine was added to each culture and the cells were harvested after 30 min of labeling. Cell fractionation, RNA extraction, and poly(A) isolation were as described in *Materials and Methods*, except that nuclear RNA labeled for 60 min with ^{32}P was added to each RNA prior to isolation of poly(A) sequences. The [^{32}P]poly(A) served as an internal marker for gel electrophoresis. (A) poly(A) from control nuclear RNA; (B) poly(A) from nuclear RNA treated with actinomycin D or 3′-deoxyadenosine; (C) poly(A) from control and drug-treated cytoplasmic RNA. (●——●) control; (×- - -×) actinomycin D; (○——○) 3′-deoxyadenosine; (|——|) internal dye marker; arrows at left are for the peak fraction of ^{32}P-labeled large nuclear poly(A) and arrows at right are for small poly(A). (The numbers on the ordinate have been multiplied by (10^{-4}.)

tinued during the chase (4). Examination of RNA sedimenting faster than 45S shows 3′-deoxyadenosine to have had little effect on the amount or the size of HnRNA synthesized. The fate of the two size classes of poly(A) during this chase is shown in Fig. 3 and Table 2. The metabolism of the two sequences is again strikingly different. Label in the small poly(A) remains essentially unchanged from that of the control (bottom of Fig. 3), which again reflects the more general lack of effect of this drug on the synthesis of HnRNA. The label in the large poly(A), however, drops more than 50% in larger HnRNA (fractions I and II) and more than 75% in the smallest HnRNA species 15 min after adding 3′-deoxyadenosine.

3′-Deoxyadenosine has been reported not to inhibit the transport of prelabeled mRNA into the cytoplasm (5). Our data are consistent with this observation in that at no time during the chase was there evidence of accumulation of poly(A)-containing RNA of the size of mRNA (fraction III) and, in fact, the decrease in large poly(A) sequences from this fraction appeared to be more rapid than from the larger HnRNA molecules.

The relatively slow disappearance of large poly(A) sequences from fractions I and II would not have been anticipated from certain estimates made in the past for the average turnover time of the large HnRNA molecules in HeLa cells, since these times have been reported to be as short as 5 min (8), although estimates as long as 30 min have also been reported (9). The 30-min value would be more compatible with these data, but, in these experiments, the possibility that 3′-deoxyadenosine is interfering with processing of HnRNA as well as polyadenylation cannot be excluded. This slow disappearance of large poly(A)-containing RNA may represent

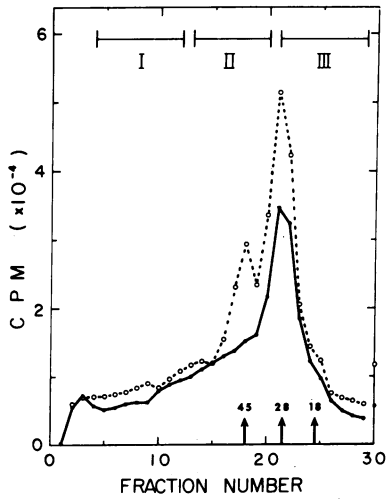

FIG. 2. Sedimentation and fractionation of nuclear RNA of control and 3′-deoxyadenosine-treated cells. Nuclear RNAs isolated from the cells after 45 min of 3′-deoxyadenosine treatment, as described in the legend of Fig. 3, were centrifuged through a 15–30% linear sucrose gradient for 13.5 hr at 18,500 rpm in a Spinco L2 65B with an SW40 rotor. After fractionation, 5-μl aliquots were counted for acid-precipitable ^{3}H. Similar differences in sedimentation patterns of nuclear RNA from treated and untreated cells shown above at 45 min were also observed at 15 and 30 min. (●——●) 3′-deoxyadenosine treated; (○- - -○) control; (|——|) fractions I, II and III pooled for further analysis as shown in Fig. 3; arrows indicate positions of 45S, 28S, and 18S RNA obtained from absorbance measurements of the control sample. (The number on the ordinate have been multiplied by 10^{-4}.)

RNA which is relatively stable pre-messenger RNA or some poly(A)-containing molecules which remain in the nucleus.

DISCUSSION

The experiments reported here clearly establish the existence of a short poly(A) sequence within the HnRNA of HeLa cells which differs from the large poly(A) sequence not only in size but in its location within HnRNA molecules and, more significantly, in its mechanism of biosynthesis. That the small poly(A) is not at the 3′-end of RNA was indicated in our earlier experiments (1) which showed that, in contrast to the large poly(A), it was not labeled by NaB^3H_4 reduction of periodate-oxidized nuclear RNA, nor was label recovered in adenosine added as a carrier to the alkaline hydrolysate of the small sequence isolated from the ribonuclease digest of [^{14}C]adenosine-labeled HnRNA.

The length of the sequence has been estimated to be in the range of 20–40 nucleotides on the basis of its electrophoretic mobility relative to the poly(A) sequence at the 3′-end of messenger RNA of yeast, which has been estimated by more rigorous methods to be 50 nucleotides long (10).

If the average length of a sequence, as well as the length of the RNA molecules containing it are known, the frequency of occurrence of the sequence per RNA molecule can be estimated from the percent of total nucleotides represented by the sequence. For example, in the case of the largest class of HnRNA molecules examined here (fraction I), which have been estimated to contain 20,000–40,000 nucleotides (11),

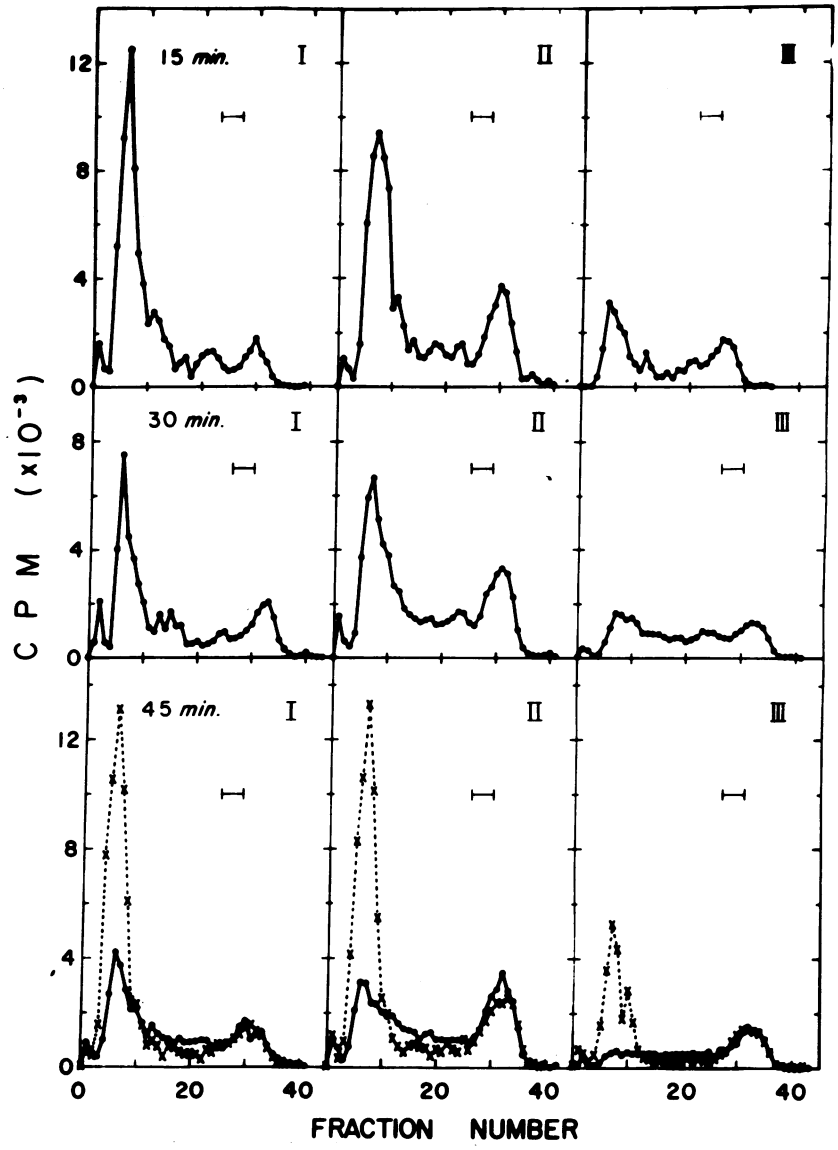

FIG. 3. Distribution of large and small poly(A) sequences in HnRNA after addition of 3′-deoxyadenosine. Cells (24×10^7) harvested in 60 ml of culture medium were separated into two spinner bottles and incubated for 20 min. After labeling for 45 min with 1.5 mCi of [^{3}H]adenosine, 3′-deoxyadenosine was added to one of the bottles. After 15, 30, and 45 min, 10 ml of culture was withdrawn and added to a tube containing 25 ml of ice-cold Earle's saline. Cells were washed and fractionated as described in *Materials and Methods*. Nuclear RNA was fractionated in a sucrose gradient as indicated in Fig. 2. Poly(A) isolation and polyacrylamide gel electrophoresis were as described in *Materials and Methods*. (●——●) poly(A) from cells treated with 3′-deoxyadenosine for 15 min (top panel), 30 min (middle panel), and 45 min (bottom panel); (×---×) poly(A) from control cells labeled for 90 min. (I, II, and III) nuclear RNA fractions described in Fig. 2.

the 200 nucleotide poly(A) sequence at the 3′-end of these molecules should represent 0.5–1% of the total RNA. In fact, however, the total poly(A) content of HnRNA molecules in this size class never accounts for more than 0.4–0.5% of the total nucleotides (1, 2), and, if only the large poly(A) is considered, the total poly(A) content would be closer to 0.3% of the total nucleotides. These experimental values are in agreement with many data showing that never more than 40%, but no less than 15%, of these HnRNA molecules bind to oligo(dT)-cellulose (12).

When a similar calculation is done for the small poly(A) sequence, estimated as 30 nucleotides long and using the fact that it represented 16% of the total poly(A) sequences of fraction I (from Fig. 3), a value of 0.08–0.15% would be expected if each HnRNA in this size range contained one small poly(A) sequence. The actual experimental finding of 0.06–0.08% based on 16% of the 0.40–0.50% of total poly(A) as small poly(A) indicates fewer than one short poly(A) sequence per HnRNA molecule. The prediction that not all HnRNA molecules contain the short poly(A) sequence is consistent with our finding that at least 15–20% of those HnRNA molecules larger than 45S do not contain small poly(A) (12), since these HnRNA molecules contain only the large poly(A). How the small poly(A) sequences are distributed in the HnRNAs that do not contain the large poly(A) is not known.

The differential effects of both actinomycin D and 3′-deoxyadenosine on the biosynthesis of the two poly(A) sequences support the proposal that the large poly(A) sequences are added after transcription of HnRNA molecules (13). This conclusion was originally based on experiments similar to those described above, which showed differential effects of each drug on HnRNA and poly(A) synthesis (3) and an apparent absence of polydeoxythymidylate sequences in DNA. The latter finding is based on the fact that neither poly(A) itself nor the poly(A) portion of messenger RNA of simian virus-40(SV40) could be hybridized to SV40 DNA (14). Poly(dT) sequences of lengths close to those of large poly(A) were not found in HeLa-cell DNA (15).

The experiments reported here provide an additional control, lacking in other experiments involving the differential effects of these drugs, in that the effects of drugs are compared on two sequences of identical composition, thus minimizing any specific effects of actinomycin D or 3′-deoxyadenosine on the polymerization of specific nucleoside triphosphates. For example, actinomycin D, even at high concentrations, does not prevent transcription of oligo(dT) templates or of crab dAT with *Escherichia coli* RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) (16).

Our experiments provide some data which relate to models (2, 13) currently proposed to account for the generation of mRNA molecules from the 3′-end of large HnRNA molecules in the nucleus which the recent demonstration that DNA complements of duck-globin mRNA hybridized to both large and small duck-HnRNA molecules supports (17). In the first place, we have defined two distinct poly(A) sequences in HnRNA molecules, only one of which appears in the cytoplasm. It thus can be assumed that the small poly(A) sequence is subjected to a nucleolytic processing similar to that for the portion of the HnRNA molecules which never leave the nucleus.

The kinetic analysis of the disappearance of the large poly(A) sequence from different size classes of HnRNA after the addition of 3′-deoxyadenosine produced two observations relevant to the model now under consideration for processing of HnRNA to generate mRNA. The observation that blocking of further adenylation of HnRNA resulted in rather rapid disappearance of poly(A)-containing RNA of the size of polysomal mRNA in the nucleus, along with the fact that no large pool of RNA of this size was found in the nucleus labeled to a steady state, suggests that the usual 15 to 20-min delay in the appearance of mRNA in HeLa cytoplasm after labeling of nuclear RNA is not likely to be a delay in transport. The transfer of pre-labeled mRNA to HeLa-cell polysomes continued in both treated and control cells during the chase period, although the amount was somewhat less in treated cells. These data, which are in general agreement with those previously reported for the effects of 3′-deoxyadenosine on mRNA transport (5), will be reported elsewhere.

On the other hand, the relatively slow disappearance of poly(A) from the large HnRNA fractions, which are assumed by the current models to be pre-messenger RNA, suggests that processing rather than transport may be the step(s) controlling the rate of appearance of mRNA on polysomes. Of course, in experiments such as these, the possibility that 3′-deoxyadenosine effects processing as well as adenylation cannot be excluded.

The differences in the metabolism of the two poly(A) sequences reported here not only support the evidence already accumulated for a post-transcriptional synthesis for the large poly(A) sequence (3, 7) but are consistent with the prediction that defined regions within HnRNA molecules have distinctive metabolic fates which their role as precursors of messenger RNA would require.

This research was supported by NIH Grant GM-18881.

1. Nakazato, H., Kopp, D. W. & Edmonds, M. (1973) *J. Biol. Chem.* **248**, 1472–1476.
2. Edmonds, M., Vaughan, M. H. & Nakazato, H. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1336–1340.
3. Darnell, J. E., Philipson, L., Wall, R. & Adesnik, M. (1971) *Science* **174**, 507–510.
4. Siev, M., Weinberg, R. & Penman, S. (1969) *J. Cell Biol.* **41**, 510–520.
5. Penman, S., Rosbash, M. & Penman, M. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1878–1885.
6. Lindberg, U. & Persson, T. (1972) *Eur. J. Biochem.* **31**, 246–254.
7. Jelinek, W., Adesnik, M., Salditt, M.,Sheiness, D., Wall, R., Molloy. G., Philipson, L. & Darnell, J. E. (1973) *J. Mol. Biol.* **75**, 515–532.
8. Soeiro, R., Vaughan, M. H., Warner, J. R. & Darnell, J. E. (1968) *J. Cell Biol.* **39**, 112–118.
9. Penman, S., Vesco, C. & Penman, M. (1968) *J. Mol. Biol.* **34**, 49–69.
10. McLaughlin, C. S., Warner, J. R., Edmonds, M., Nakazato, H. & Vaughan, M. H. (1973) *J. Biol. Chem.* **248**, 1466–1471.
11. Molloy, G. R., Thomas, W. L. & Darnell, J. E. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3684–3688.
12. Nakazato, H. & Edmonds, M. (1973) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. XII (E), in press.
13. Darnell, J. E., Jelinek, W. R. & Molloy, G. R. (1973) *Science* **181**, 1215–1221.
14. Philipson, L., Wall, R., Glickman, G. & Darnell, J. E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2806–2809.
15. Birnboim, H. C., Mitchell, R. E. & Strauss, N. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2189–2192.
16. Goldberg, I. H., Rabinowtiz, M. & Reich, E. (1962) *Proc. Nat. Acad. Sci. USA* **48**, 2094–2101.
17. Imaizumi, T., Diggelmann, H. & Scherrer, K. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1122–1126.