Addition of Poly(A) to Nuclear RNA Occurs Soon after RNA Synthesis

M. SALDITT-GEORGIEFF, M. HARPOLD, S. SAWICKI, J. NEVINS, and J. E. DARNELL, JR.
The Rockefeller University, New York 10021. Dr. Harpold's present address is the University of California School of Medicine, Los Angeles, California 90033. Dr. Sawicki's present address is the Medical College of Ohio, Toledo, Ohio 43699.

ABSTRACT A kinetic analysis of the appearance of [^{3}H]uridine label in RNA sequences that neighbor poly(A), as well as the incorporation of [^{3}H]adenosine label into both the RNA chain and the poly(A) of poly(A)-containing molecules, shows that poly(A) is added within a minute or so after RNA chain synthesis in Chinese hamster ovary cells and HeLa cells. Previous conclusions by several groups (5–7) that poly(A) might be added as long as 20–30 min after RNA synthesis appear to be in error, and the present conclusion seems much more in line with several different types of recent studies with specific mRNAs that suggest prompt poly(A) addition (13–16).

Segments of polyadenylic acid were discovered in RNA samples from liver cells in the 1960s (for review see reference 1). The addition of poly(A) to nuclear molecules, the hnRNA, before its appearance in the cytoplasm as part of mRNA was the first demonstration of the several steps that occur in the "processing" of nuclear RNA to make mRNA (2, 3).

Early studies on poly(A) metabolism concluded that although poly(A) could be added within a few minutes to nuclear RNA (4), the addition was thought to occur possibly as long as 20–30 min after a nuclear mRNA precursor was synthesized (5–7). As the nuclear formation of specific cell mRNAs and mRNAs from DNA viruses have been studied over the past several years, a different conclusion has been suggested.

In both Ad-2 transformed cells and early lytic infection, certain virus-specific mRNAs can be synthesized, polyadenylated, spliced, and transported to the cytoplasm all in 10 min or less (8, 9). In addition, hemoglobin mRNA in mouse cells can also be synthesized and processed, including poly(A) addition, within 10 min or less (10, 11). In late adenovirus mRNA formation, one of the best studied cases of mRNA formation (for review see reference 12), poly(A) addition occurs so promptly that the primary transcript may not even be finished before nucleolytic cleavage and poly(A) addition occurs (13). Splicing of the late Ad-2 primary transcript then occurs after poly(A) addition. Likewise, it has been shown for mRNA production from two early transcription units that the great majority if not all poly(A) addition precedes splicing (14). For a number of other virus, as well as cell transcription units, nuclear, unspliced poly(A)-containing molecules can be detected (15–17), supporting the general conclusion that splicing occurs after poly(A) addition.

All of this recent work suggests that poly(A) addition is a prompt rather than a late event in mRNA biogenesis. We have, therefore, reexamined whether the earlier conclusion of delayed poly(A) addition in general nuclear RNA metabolism was proper. It is not. Several different experiments involving total cell poly(A) synthesis indicate that the maximum delay is perhaps 1 or 2 min before the majority of poly(A) addition occurs in new nuclear RNA molecules.

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells and HeLa cells were grown in suspension and labeled as described (18, 19). For convenience nuclear RNA was extracted in a neutral buffer as described for cytoplasmic RNA rather than in an acetate buffer as previously described (20). After the nuclei were lysed and incubated for 2 min at 4°C in a high-salt buffer containing DNAse 5×10^7 nuclei/milliliter, they were lysed in 0.05 M $MgCl_2$, 0.5 M NaCl, and 0.01 M Tris, pH 7.4, containing 100 μg/ml Worthington RNAse-free DNAse treated with iodoacetate (Worthington Biochemical Corp., Freehold, N. J.) as described (22), an equal volume of ETS (0.01 M EDTA, 0.01 M Tris, and 0.2% SDS) plus sufficient EDTA to make the final concentration 0.01 M and sufficient SDS to make final concentration 0.5% was added. The mixture was then extracted with an equal volume of phenol plus an equal volume of chloroform as described for cytoplasmic samples; RNA was collected by ethanol precipitation from the aqueous phase.

Poly(U)-Sepharose chromatography of poly(A)-containing mRNA molecules or poly(A) segments has been described (20) as was the gel electrophoretic analysis of RNA samples (19). Infection of HeLa cells with adenovirus and assay of adenovirus-specific RNA by hybridization has been described (21).

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RESULTS

Kinetics of Label Appearance in Poly(A) Containing mRNA: [^{3}H]Uridine Labeling of Nuclear RNA

The simplest experiment to measure the time of addition of poly(A) to nuclear molecules in cultured CHO cells is recorded in Fig. 1. Cells treated with a low dose of actinomycin to suppress rRNA synthesis were labeled with [^{3}H]uridine for 4 min, and six samples were removed within that short time. Nuclear RNA was prepared and broken by controlled alkali treatment to an average size of ~500 bases (23; determined by gel electrophoresis, data not shown). Fragments of labeled nuclear RNA of this length would contain poly(A) of ~200 to 250 bases (19) that would be unlabeled, plus ~200 to 300 bases that could contain labeled uridylate residues depending upon the length of time between RNA synthesis and poly(A) addition. (Cytidylate residues do not become labeled because of pyrimidine interconversion in such brief exposures; 24.) The RNA fragments were passed through poly(U)-Sepharose and the total radioactivity in poly A^+ and poly A^- fragments was determined. The relative rate of accumulation of radioactivity in poly(A)$^+$ and poly(A)$^-$ fractions was almost identical (Fig. 1). Perhaps the poly(A)$^-$ fraction rose 15–30 s faster than the poly(A)$^+$ fraction. This result indicates a delay of 30 s or less between the time of synthesis of an hnRNA segment and the acquisition of any poly(A) tail it is going to acquire. If most new [^{3}H]uridine-labeled molecules waited for ~10 min before acquiring poly(A), then the poly(A)$^+$ fragments should have been much more slowly labeled than the total poly(A)$^-$ fragments.

Moreover, the proportion of [^{3}H]uridine label in the poly(A)$^+$ fraction in the experiment of Fig. 1 agrees with the conclusion that the majority of the [^{3}H]uridine-labeled chains that will become associated with poly(A) will have done so within a short time. For example, after long label times about one-fifth of the hnRNA molecules are thought to become polyadenylated (4, 20) and the average chain size of completed hnRNA in both HeLa cells and CHO cells is ~5,000 bases (25–27). If the "nascent"-labeled RNA in Fig. 1 has in fact acquired as much poly(A) as it ever will, then roughly one-fifth of the whole molecules should be poly(A)$^+$. Because the nascent RNA represents fragments containing 200–300 bases of RNA (in addition to unlabeled poly[A]) then about one-fifth of 250/5,000 or ~1% of the [^{3}H]uridine label would be in poly(A)$^+$ fragments if little or no lag occurred before poly(A) addition. Approximately 1–1.5% of the [^{3}H]uridine label was, in fact, present in poly(A)$^+$ fragments (Fig. 1).

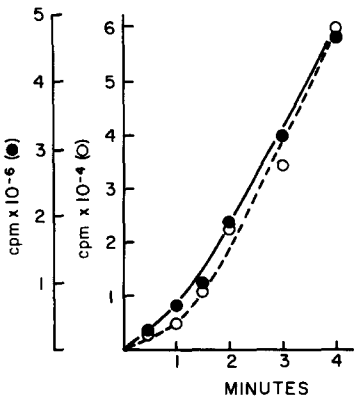

FIGURE 1 Rate of labeling of total mRNA compared with sequences in neighboring poly(A). CHO cells growing in suspension were harvested at 37°C and resuspended at 2 × 10^6 cells/ml in Eagle's medium with 0.05 μg/ml of actinomycin D to suppress rRNA formation. After 25 min, [^{3}H]uridine (25 mCi/μmol; 200 μc/ml) was added and samples (2 × 10^7 cells/sample) were taken at indicated times and total nuclear RNA extracted. The RNA was treated for 30 min at 0°C with 0.2 M NaOH in ETS (0.01 M EDTA, 0.01 M Tris, and 0.12% SDS) as described (23) to reduce the RNA chains to an average length of 500 ± 250 nucleotides (confirmed by gel analysis, data not shown; see Fig. 2 in reference 23). Each RNA sample was then subjected to poly(U)-Sepharose chromatography (20) and portions of the bound (poly(A)$^+$ ○) and unbound (poly(A)$^-$ ●) fractions assayed for acid-precipitable radioactivity.

[^{3}H]Adenosine Labeling of Nuclear and Cytoplasmic RNA

A second experiment with [^{3}H]adenosine was performed in which cells were labeled for both short and long periods of time and again the 3′ terminal poly(A)-containing fragments isolated (Table I). In this experiment the selected terminal fragments of ~500 bases should contain label in the new poly(A) as well as label in RNA chains. If poly(A) were added mainly to preexisting chains then the fraction of [^{3}H]adenosine label in new poly(A) compared with label in the RNA chain should first be very high and then decline. The fraction of [^{3}H]adenosine label in poly(A) compared with the neighboring sequences in the nuclear RNA was, however, almost exactly the same from 10 through 40 min of labeling. Even after only 1 min of labeling, the ratio of label in poly(A) to label in RNA chains was less than twice that of the later label times, indicating that >1/2 of the chains that would acquire poly(A) had done so in 1 min. Again, it appears that only a very short lag occurs between the synthesis of an RNA chain and the addition of its poly(A).

In the experiments of Mendecki et al. (5) and of Perry et al. (6), the percentage of total [^{3}H]adenosine label in cytoplasmic poly(A)-containing RNA that was in poly(A) or in the mRNA chain was determined after cells were labeled for various periods of time. In those experiments, during the first 15–30 min the percentage of recovered label in poly(A) compared with the remainder of the mRNA chain was higher than for molecules labeled for longer times. Both groups of workers at

TABLE I

Labeling of Poly(A) and Neighboring Sequences

Label time	cpm in Poly(A)	cpm in RNA chain	Ratio of cpm Poly(A)/RNA chain
min			
1	440	60	7.3
10	1,700	360	4.7
20	2,240	459	4.9
30	3,400	800	4.3
40	5,700	1,270	4.5

CHO cells were collected and resuspended at 37°C, and [^{3}H]adenosine (20 mCi/μmol; 100 μc/ml) was added for the indicated times. Nuclear RNA was isolated and RNA fragments were prepared as indicated in Fig. 1. The poly(A)$^+$ fragments were selected and precipitated with ethanol. The fragments were redissolved in 1 ml of 0.01 M Tris, 0.01 M EDTA and digested for 45 min at 37°C with 10 U/ml of T1 RNAse (20). The samples were then passed through poly(U)-Sepharose and radioactivity monitored in the bound and unbound fractions. The unbound fraction represented label in the sequences neighboring poly(A). The bound material (which was eluted in buffer with 50% formamide; see reference 20) represented poly(A).

that time took this as evidence of addition in the nucleus of new poly(A) to older, preexisting molecules. However, a number of experiments since that time have cast doubt on this possible explanation. Brawerman and Diez (1, 28) demonstrated the occurrence of terminal labeling of preexisting cytoplasmic poly(A). This end-labeling increases the fraction of a brief [^{3}H]adenosine label found in cytoplasmic poly(A) compared with the internal residues of cytoplasmic mRNA molecules (19). The terminal addition, however, occurs to all cytoplasmic poly(A)-containing RNA, which consists mainly of shortened, older poly(A) chains (29, 30). Thus, the end-labeling results in a heterodisperse pattern of [^{3}H]adenosine-labeled poly(A) detected by gel electrophoretic analysis in molecules from briefly labeled cells (19). In contrast, the new poly(A) is discrete in size, ~230 ± 30 bases.

With these ideas in mind CHO cells were labeled with [^{3}H]adenosine for various periods, and the poly(A)-containing cytoplasmic RNA collected by adsorption and elution from poly(U)-Sepharose. After digestion of the samples with RNAse and reselection of the poly(A) fragments, gel electrophoresis of the poly(A) segments in each sample was performed. The sample from the shortest label time (7.5 min) showed ~25% of the label associated with a heterogeneous class of poly(A) segments smaller than the newly arrived 200–250 nucleotide poly(A) segments from the nucleus (Fig. 2). By comparison, the poly(A) segments from a longer label time (45 min) showed almost all (>90%) of the label in poly(A) segments of about the same size as the newly synthesized nuclear poly(A) segments.

When the amount of label in the poly(A) segments of the cytoplasmic molecules was corrected for the terminal addition, which produces the heterogeneity in the electrophoretic profile, then the accumulation curves of [^{3}H]adenosine label in new poly(A) segments and within the new RNA chains of poly(A)-containing molecules show an almost identical increase with time from 25 to 45 min (Fig. 3). There is still a slightly faster appearance of label in poly(A) than in the body of the RNA chain during the first 15 min. However, as seen in the insert to Fig. 3, the ATP pool requires about 10 min to reach maximum specific activity and this delay could account for the slightly faster labeling of poly(A) in the first two points of Fig. 3. Because the poly(A) is the last portion of a nuclear poly(A)-containing molecule to be made, any distant 5′ regions of a nuclear precursor that eventually appear in mRNA will have been made a few seconds to a few minutes before the poly(A). During the period of rapid rise of the acid-soluble pool, this fact will cause a slight delay in the labeling of the body of the mRNAs as compared with the poly(A).

Most important, however, in the curves of Fig. 3 is the fact that during the majority of the accumulation (from 25 through 45 min), there was a fourfold increase in [^{3}H]adenosine label in the poly(A) as well as in the RNA chain. If a significant fraction of nuclear molecules that received poly(A) waited ~10 min after synthesis, the curves of appearance of label in poly(A) and RNA chain would not rise together during this interval.

Effects of Actinomycin on Poly(A) Synthesis

One of our early experiments that suggested a lag before the addition of some of the poly(A) segments involved the use of actinomycin (2, 4, 31). This drug stops the synthesis of hnRNA, but for a brief period allows presumably complete hnRNA molecules to acquire poly(A) within the nucleus. In one such experiment (31) cells were pulsed with [^{3}H]adenosine for 2 min

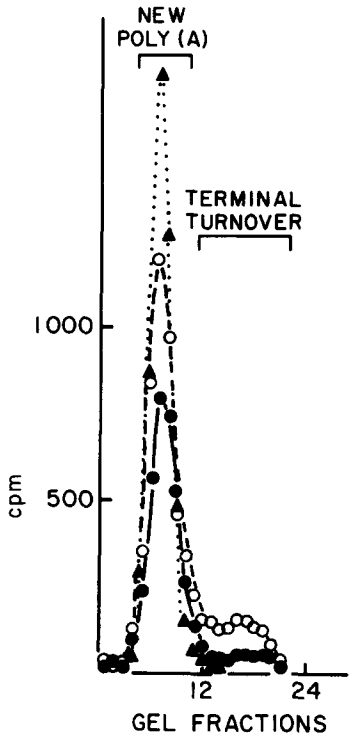

FIGURE 2 Electrophoretic analysis of cytoplasmic poly(A) compared with nuclear poly(A). CHO cells were collected and labeled with [^{3}H]adenosine as described in Fig. 1 and Table I. No actinomycin was used in the experiment. At indicated times cells were fractionated into nuclear and cytoplasmic fractions and the poly(A)$^+$ cytoplasmic RNA, prepared as in Harpold et al. (18). A portion of each sample was subjected to complete T1 RNAse digestion and poly(U)-Sepharose chromatography to collect poly(A) for gel electrophoretic analysis (19). Poly(A) from a portion of nuclear RNA from the 7.5-min sample was also selected. The electrophoretic patterns for two of the six cytoplasmic samples is shown in this figure. The poly(A) of homogeneous size (fractions indicated) was used to estimate accumulation of new poly(A) that is plotted in Fig. 3. ▲, Nuclear poly(A), 7.5 min. ○, Cytoplasmic poly(A), 7.5 min. ●, Cytoplasmic poly(A), 45 min.

after 8 min of exposure to actinomycin to measure what was thought to be synthesis of new poly(A) (23). 15% as much [^{3}H]adenosine was incorporated in 2 min into poly(A) as in control cells. We have more recently found (19) that nuclear poly(A) is also subject to terminal addition as is the cytoplasmic poly(A). Thus, the end addition reaction might continue in actinomycin. This was tested by inhibiting cells with actinomycin for various times and then labeling cells for 3 min with [^{3}H]adenosine. The ratio of label in AMP to terminal adenosine in the labeled poly(A) can be used as a more accurate guide to continued synthesis of new poly(A) segments (Table II). The AMP to adenosine ratio in control poly(A) segments was ~60:1, indicative of a mixture of labeling caused by new synthesis and terminal addition. (For example, the poly(A) segment 250 bases long with a terminal A_{OH}, plus three chains with ~two AMPs and 1 A_{OH} terminally labeled would give a ratio of 60:1; see reference 19.) However, the ratio declined in the actinomycin-treated samples to levels comparable to those observed in the total absence of new poly(A) synthesis, a state achieved by the addition of the drug 3′ deoxyadenosine, cordycepin. This drug appears to stop all poly(A) synthesis (2, 3) but does not stop terminal turnover (19, 27) on either nuclear or cytoplasmic poly(A). The ratio of labeled AMP to adenosine in the

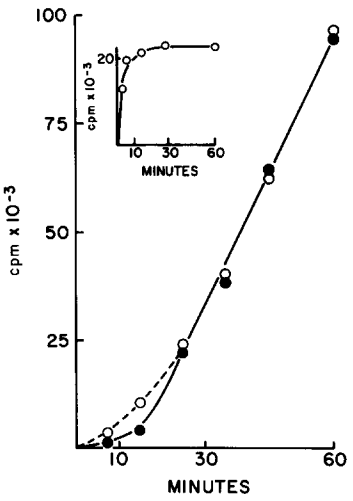

FIGURE 3 Accumulation of labeled poly(A) and label in RNA chains of cytoplasmic poly(A)$^+$ RNA. Samples from the experiment described in Fig. 2 were used. Radioactivity in poly(A) represents that in newly arrived, ~200–250 base segments (see Fig. 2) and label in the RNA chain was assayed after T1 RNAse digestion as described in Table II. The insert shows the appearance of label in ATP in 5 × 10^6 cells as a function of time. Acid-soluble pools were prepared and analyzed by thin-layer chromatography as described (24). O, Poly(A). ●, mRNA chain terminus.

TABLE II

Terminal Labeling of Nuclear Poly(A) in Actinomycin

Experiment		Ratio
		Adenosine + AMP/ Adenosine
	min	
1 Control, no drugs		61
Actinomycin	1	41
Actinomycin	2	26
Actinomycin	4	17
Actinomycin	6	12
2 Control, no drugs		54
Actinomycin	2	21
3′ Deoxyadenosine	2	13

HeLa cells were collected at 3 × 10^6 cells/ml and labeled for 3 min with [^{3}H]adenosine (see Table 1) after no treatment or after exposure of 25 μgm/ml of actinomycin D or 100 μgm of 3′ deosyadenosine. The labeled poly(A) segments from each sample were collected after T1 RNAse digestion and electrophoresis (19) and subjected to alkaline degradation. The hydrolyzed samples were then analyzed for radioactivity in the adenosine 3′ termini and AMP internal residues by electrophoresis as previously described (19). The amount of adenosine recovered in the various samples was from 350 to 500 cpm and the amount of AMP from 5,000 to 50,000 cpm.

poly(A) segments of actinomycin-treated cells approaches that of the poly(A) segments from 3′ dA-treated cells within a few minutes. This suggests that already within a few minutes of actinomycin treatment no new synthesis of poly(A) is carried out, and the only [^{3}H]adenosine label added to nuclear poly(A) after 3 min of actinomycin treatment is terminal labeling.

An experiment examining the addition of poly(A) to adenovirus sequences after actinomycin treatment concurs with this last interpretation. Cells late in adenovirus infection were labeled for 1 min with [^{3}H]adenosine either with no actinomycin in the culture or after various periods of exposure to the drug. Fig. 4 shows that as little as 1 min of actinomycin exposure stopped total adenovirus RNA synthesis and poly(A) synthesis also was considerably decreased. A lag time of

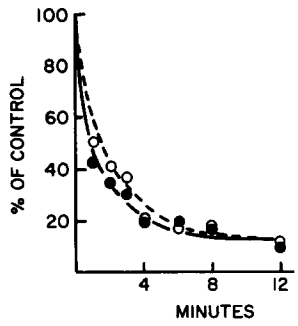

FIGURE 4 Adenovirus-specific RNA and poly(A) synthesis after actinomycin D treatment. HeLa cells at 16 h postinfection were concentrated to 2 × 10^6 cells/ml in warm medium (21). 5-ml samples received either no actinomycin D or 10 μg/ml of actinomycin D for varying lengths of time up to 12 min. [^{3}H]Adenosine was then added to each sample for 90 s. The nuclear RNA from each sample was purified and hybridized to total adenovirus DNA bound to nitrocellulose filters. After washing the hybrids, each filter was exposed separately to RNAse A and T1 for 30 min at 37°C in 0.3 M NaCl, 0.3 M Na citrate (2 × SSC). The supernatant fluid was TCA-precipitated and counted (Ad-2 specific poly(A)). The filters were washed, dried, and counted (Ad-2 specific RNA). O, Poly(A). ●, AD-2RNA.

poly(A) addition of no longer than 30–60 s can be inferred from this experiment for a specific set of nuclear mRNA precursors. This brief lag agrees with the work in the first section of this paper suggesting only a short lag time for poly(A) synthesis and also with experiments of a different design showing prompt addition of poly(A) to transcripts of the major late adenovirus transcription unit (13).

DISCUSSION

The evidence cited in this paper strongly favors the interpretation that if poly(A) is to be added to a primary transcript in the cell nucleus this decision is made very soon after synthesis of the RNA. We had earlier been struck by the speed of this decision even late in adenovirus infection where one in five possible poly(A) sites seems to be chosen almost simultaneously with transcription of RNA from the major late promoter (13). As we suggested for that situation, perhaps the RNA synthetic complex carries with it the capacity to recognize and act on a poly(A) site. If there were poly(A) recognition factors that accompany RNA polymerase II then ~75% of the nuclear transcripts that never become polyadenylated (4, 20) are transcribed by a different complex lacking poly(A) recognition capacity or these transcription units lack poly(A) sites. What remains unknown, of course, is whether specific nuclear RNA molecules that do not become polyadenylated in one cell at one stage in differentiation ever become polyadenylated. These molecules could either perform their function, for example as poly(A)$^-$ mRNAs (32–34), or might under other circumstances have poly(A) added. Whatever the nature of the poly(A)$^-$ fraction, it appears that molecules destined to acquire poly(A) do so very promptly.

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