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Addition of Polyadenylate Sequences to Virus-Specific RNA during Adenovirus Replication

(HeLa cells/cordycepin/DNA–RNA hybridization/"early" and "late" RNA)

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ABSTRACT **Adenovirus-specific nuclear and polysomal RNA, both early and late in the infectious cycle, contain a covalently linked region of polyadenylic acid 150–250 nucleotides long. A large proportion of the adenovirus-specific messenger RNA contains poly(A). As revealed by hybridization experiments, the poly(A) is not transcribed from adenovirus DNA. Furthermore, an adenosine analogue, cordycepin, blocks the synthesis of poly(A) and also inhibits the accumulation of adenovirus messenger RNA on polysomes. Addition of poly(A) to viral RNA may involve a host-controlled mechanism that regulates the processing and transport of messenger RNA.**

A unit of polyadenylic acid [poly(A)]* of about 150–250 nucleotides is covalently linked, probably in a terminal position, to both heterogenous nuclear RNA (HnRNA) and messenger RNA (mRNA) in eukaryotic cells (1–4). Poly(A) sequences have also been found in vaccinia mRNA molecules (5). The cellular poly(A) segment originates in the nucleus and appears to be added after transcription, since the inhibition of total labeling of HnRNA and mRNA by actinomycin D is considerably greater than the inhibition of the labeling of poly(A) in these classes of RNA (6). Furthermore, the addition of poly(A) appears to be necessary for the nuclear processing of HnRNA to mRNA, or for the transport of some mRNA molecules from the nucleus to the cytoplasm, since cordycepin (3′-deoxyadenosine), which blocks synthesis of poly (A) in both HnRNA and mRNA, inhibits the accumulation of total label in mRNA on polysomes (6, 7).

The present study demonstrates the presence of poly(A) in adenovirus-specific nuclear RNA and polysomal RNA during virus replication. The poly(A) must in this case be added after transcription, because the virus DNA does not contain sequences that hybridize with poly(A). It appears, therefore, that during adenovirus multiplication a preexisting host mechanism for the post-transcriptional modification of nuclear RNA is used to process virus mRNA.

MATERIAL AND METHODS

Cell Cultures and Virus Infection. A strain of HeLa cells capable of supporting the growth of adenovirus to a high titer was kindly provided by Dr. J. V. Maizel. Cultures were grown in suspension in Eagle's minimal essential medium (8), with 5% fetal-calf serum. Adenovirus Type 2 was propagated (9) by infection with a multiplicity of 2000 plaque-forming units per cell at a density of 2–3 × 10^5 cells/ml. Virus was purified from infected cells 48 hr after infection. Viral DNA was extracted from purified virus (10).

Preparation of RNA. [^{3}H]Uridine or [^{3}H]adenosine, (25 and 24 Ci/mmol, respectively) were used to label concentrated suspensions of infected cells (4 × 10^6/ml, 0.1–1 mCi/ml). Late adenovirus RNA was obtained by labeling of infected cells 16–18 hr after infection, while early adenovirus RNA was prepared by labeling of infected cells 2–6 hr after infection. To insure that only early RNA was produced, cells to be labeled between 2 and 6 hr were treated with cycloheximide (25 μg/ml) from 1 hr after infection so that no viral DNA and, consequently, no late viral RNA was produced (11). Cytoplasm of infected cells was obtained by breakage of cells with the nonionic detergent, NP40. Polysomes were isolated either by centrifugation through 2 M sucrose (12) or by zonal sedimentation. Polysomal RNA was released by EDTA and recovered from a second zonal sedimentation (13). Polysomal and nuclear RNA were extracted by the phenol–sodium dodecyl sulfate procedure (14). All RNA preparations were precipitated twice with 2 M LiCl and treated with DNAse before experimentation (15). Labeled RNA preparations were stored under ethanol at −20°C.

DNA–RNA Hybridization. Hybridization and elution of virus-specific hybrids has been described in detail (16). Unbroken adenovirus-specific RNA was selected in 30% formamide (v/v)–0.3 M NaCl–10 mM EDTA–10 mM TES-[*N*-tris(hydroxy-methyl) methyl-2-aminoethane sulfonic acid] (pH 7.4) at 45°C. Exhaustion hybridization was performed in the same buffer without formamide at 65°C for 24–48 hr, followed by a wash and RNase treatment of the filters.

Adenovirus-specific sequences were released from viral DNA–RNA hybrids by boiling in 10 mM EDTA (pH 7.5) for 15 min.

Determination of Poly(A) Content. Virus RNA selected by hybridization and elution from adenovirus DNA was treated with 20 μg/ml of DNase in 0.1 M NaCl–1 mM $MgCl_2$–10 mM Tris (pH 7.4) for 30 min at 37°C, followed by digestion with pancreatic RNase (2 μg/ml) and T1 RNase (10 units/ml) in 0.2 M NaCl–10 mM EDTA–10 mM Tris (pH

Abbreviation: HnRNA, heterogeneous nuclear RNA.

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* While these RNA segments are at least 90% adenylic acid (1, 2, 6), and one report claims 99% (2), it is not strictly proven that they consist entirely of adenylate residues. For convenience, they will be described in this paper as poly(A).

7.4) for 30 min at 37°C. RNase-resistant RNA was precipitated with ethanol, redissolved, and passed through Millipore filters (4). The bound poly(A) was eluted with 0.1 M Tris (pH 8.8)–10 mM EDTA–0.5% sodium dodecyl sulfate at 4°C overnight. Poly(A) was measured as a 7–10S species in 10% acrylamide gels (6).

Hybridization of Poly(A). Poly(A) from infected and uninfected cells, isolated by polyacrylamide electrophoresis (6), was hybridized at 50 and 65°C in 0.3 M NaCl–10 mM EDTA–10 mM TES (pH 7.4), and at 45°C in the same solution containing 30% formamide. RNase treatment of bound poly(A) was done with 0.1 unit/ml of T2 RNase in 0.9 M NaCl–10 mM EDTA–10 mM acetate buffer (pH 4.5) (5).

Selection of High Molecular Weight RNA Containing Poly(A). Two methods were used to specifically select RNA molecules containing poly(A) sequences. First, poly(U)–cellulose columns were prepared as described by Britten (17) and modified by Kates (5). RNA was filtered through them in 0.1 M NaCl–10 mM Tris (pH 7.4) at 4°C and eluted at 45°C with 10 mM Tris (pH 7.4). Alternatively, adsorption to Millipore filters (14) was done in 0.5 M KCl–1 mM $MgCl_2$–10 mM Tris (pH 7.4), treated with diethyl pyrocarbonate (Baycovin). Bound RNA was eluted by stirring in 0.1 M Tris (pH 8.8)–10 mM EDTA–0.5% sodium dodecyl sulfate at room temperature for 5–6 hr.

RESULTS

Selection of adenovirus-specific RNA sequences

In order to detect sequences covalently linked to viral RNA, though not necessarily arising from transcription of the viral genome, hybridization of viral-specific RNA must be performed under conditions that minimize breakage of the molecules. Hybridization in formamide at low temperature was used to isolate unbroken RNA molecules (16). Table 1 shows that hybridization of polysomal RNA from infected cells in 30% formamide at 45°C without subsequent RNase treatment gave comparable amounts of adenovirus-specific hybrid as the more common procedure of hybridization at 65°C,

TABLE 1. *Comparison of hybridization of adenovirus RNA with and without formamide*

RNA sample	Filters	Input cpm	cpm Hybridized* 45°C, Formamide − RNase	cpm Hybridized* 2 × TES 65°C + RNase
Early Polysomal RNA 2–6 hr	Adenovirus DNA	42,000	9,870	7,310
	Blank		350	51
Late Polysomal RNA 16–18 hr	Adenovirus DNA	36,000	27,680	18,300
	Blank		410	62
Uninfected cells Polysomal RNA	Adenovirus DNA	106,000	150	12
	Blank		15	11

* Hybridization to 20 μg of adenovirus-DNA filters for 24 hr and exhaustively hybridized for an additional 24 hr.

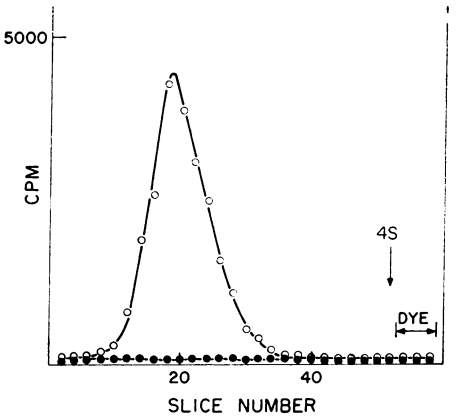

FIG. 1. Polyacrylamide gel electrophoresis of poly(A) in early adenovirus-specific RNA.

Polysomal RNA, labeled with [^{3}H]adenosine (O——O) or [^{3}H]uridine (●——●) was purified and digested as described in *Methods*. The digests were subsequently selected on Millipore filters and analyzed on 10% polyacrylamide gels.

followed by RNase treatment. Polysomal RNA from uninfected cells did not hybridize to adenovirus DNA, since 0.1% of the input was nonspecifically bound to viral DNA with the formamide method.

Poly(A) in adenovirus-specific RNA

Adenovirus-specific nuclear and polysomal RNA labeled with [^{3}H]uridine or [^{3}H]adenosine, was selected on adenovirus DNA. Hybrid eluates were digested with DNase and RNase. Their poly(A) content, determined by polyacrylamide gel electrophoresis, revealed an RNase-resistant species of 150–250 nucleotides in the adenosine-labeled adenovirus-selected RNA (Fig. 1). No RNase-resistant uridine label was observed in the region of the adenosine-labeled poly(A) peak.

Adenovirus-selected early or late polysomal RNA contained 6–8% of the adenosine label in the poly(A) sequences, while about 2% of the adenosine label in early or late viral-specific

TABLE 2. *Poly(A) content of adenovirus-specific RNA*

RNA	Hours	Input cpm	Total cpm bound to adenovirus DNA*	% Virus-specific RNA as poly(A) in gel (%)
A-Labeled				
Nuclear	2–6	2.5×10^7	8.8×10^5	1.4
	16–18	4.1×10^5	3.5×10^4	2.0
Polysomal	2–6	1.7×10^7	1.7×10^6	6.1
	16–18	9.1×10^5	2×10^5	8.4
U-Labeled				
Nuclear	2–6	3.8×10^7	1.3×10^6	<0.1
	16–18	1×10^6	9.8×10^4	<0.1
Polysomal	2–6	1.3×10^7	1.5×10^6	<0.1
	16–18	1.15×10^6	3.4×10^5	<0.1

*Hybridization to 40 μg of adenovirus DNA in 16–18 hr at 45°C in 30% formamide. Adenosine- and uridine-labeled adenovirus-selected polysomal RNA were assayed for poly(A) content as in Fig. 1.

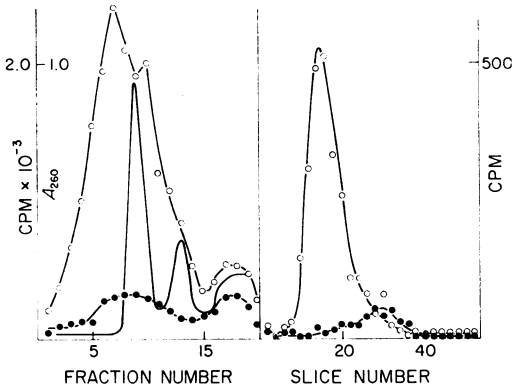

FIG. 2. The effect of cordycepin on polysomal RNA and poly(A) during adenovirus replication. About 4×10^8 HeLa cells were infected with adenovirus. At 16 hr after infection, the culture was divided and half was treated with cordycepin (50 μg/ml) for 10 min. Both portions were then labeled with [^{3}H]adenosine for 45 min (1 mCi in 20 ml). Polysomes were isolated by zonal sedimentation, treated with 10 mM EDTA, and sedimented through a second sucrose gradient containing EDTA (13).

Left panel: The continuous line represents absorbance at 260 nm, which was identical for the treated and control cultures. The two peaks represent 50S and 30S ribosomal subunits. Radioactivity: O——O, control; ●——●, cordycepin-treated.

Right panel: Polyacrylamide gel pattern of poly(A) from adenovirus-specific RNA. Fractions 4–13 from the *left panel* were precipitated with ethanol, extracted with phenol, selected on adenovirus DNA, and then assayed for poly(A). A total of 5420 cpm was recovered in poly(A) from adenovirus-specific RNA of untreated cells, amounting to 10% of the total virus-specific RNA, compared with at most 230 cpm in the cordycepin-treated cells, or less than 1% of this RNA. Radioactivity: O——O, control; ●——●, cordycepin-treated.

nuclear RNA was recovered as poly(A) (Table 2). Poly(A) with the same electrophoretic mobility from nuclear and polysomal RNA of uninfected cells is composed of more than 90% adenylic acid (6). The poly(A) from adenovirus-selected RNA contained between 81–95% adenylic acid and was completely sensitive to T2 RNase, an enzyme that preferentially attacks linkages between adenylic acid (5). Thus, the poly(A) associated with adenovirus RNA probably represents the same adenylate-rich species recovered from uninfected cell RNA.

TABLE 3. *Hybridization of poly(A) to adenovirus and HeLa DNA*

	cpm Hybridized					
	−T2 RNase			+T2 RNase		
Filter	45°C	50°C	65°C	45°C	50°C	65°C
	Formamide			*Formamide*		
Adenovirus DNA, 20 μg	32	69	42	20	21	22
Blank	69	63	37	28	50	20
HeLa DNA, 20 μg	39	2826	43	29	381	29
Blank	44	82	41	21	42	32

Poly(A) (4800 cpm vial), prepared on acrylamide gels from uninfected HeLa cells, was hybridized for 18 hr to HeLa- or to adenovirus-DNA. Poly(A), about 10^5 cpm/mg.

The origin of the poly(A) sequences in adenovirus-specific RNA

In order to determine whether adenovirus DNA contained sequences that are transcribed into poly(A), an attempt was made to hybridize poly(A) to viral DNA under conditions known to allow the binding of poly(A) with poly(T) (18), as well as the binding of poly(A) to vaccinia DNA (5). Even before T2 RNase digestion, labeled poly(A) from uninfected HeLa cells failed to bind at 50°C to adenovirus DNA (Table 3). Poly(A) derived from adenovirus-specific RNA also did not hybridize to viral DNA. In contrast, a small amount of poly(A) did bind to HeLa DNA at 50°C, even after digestion with T2 RNase. No binding of poly(A) to either adenovirus or HeLa DNA was observed under the incubation conditions routinely used in DNA–RNA hybridization. From these results, it appears that the poly(A) sequences in adenovirus RNA do not arise from the transcription of viral DNA.

Fraction of adenovirus polysomal RNA molecules containing poly(A)

To assess whether the majority of individual adenovirus mRNA molecules contained poly(A), early and late polysomal RNA from adenovirus-infected cells were exposed to Millipore filters (4) or poly(U)–cellulose columns (5, 17). Bound- and unbound-RNA fractions were then separately hybridized to adenovirus DNA. Table 4 shows that around 65–80% of the adenovirus-specific early and late polysomal RNA was selected on Millipore filters as if it contained poly(A). The same distribution of binding was found by Lee *et al.* (4) for sarcoma cell mRNA. When late viral-specific RNA from polysomes was analyzed by chromatography on poly(U)–cellulose, it was found that about 60% of the adenovirus-specific mRNA was retained by the column (Table 5). It should be emphasized that single breaks (nicks) in the RNA molecules would reduce the actual values, suggesting that the great majority, and perhaps all, of the adenovirus-specific polysomal RNA contains a poly(A) segment.

The effect of cordycepin on adenovirus-specific RNA

It has been shown that cordycepin (3′-deoxyadenosine) reduced the amount of 7–10S poly(A), both in nuclear and polysomal RNA from uninfected cells (6). Since the majority of adenovirus-specific polysomal RNA appears to contain poly(A), cordycepin might also be expected to drastically

TABLE 4. *Adenovirus-specific sequences in Millipore-selected polysomal RNA*

RNA	Total input cpm	Adenovirus-specific cpm*		Total cpm of adenovirus RNA bound (%)
		Bound and eluted	In filtrate	
Polysomal RNA 2–6 hr	370,000	49,700	23,700	67.8
Polysomal RNA 16–18 hr	125,000	26,750	6,340	81.0

Adenovirus-specific polysomal RNA labeled late after infection with [^{3}H]adenosine was exposed to Millipore filters (4). Bound and unbound fractions were then assayed for their content of adenovirus-specific RNA by exhaustive exposure to adenovirus DNA.

* After 48 hr of exposure to 80 μg of DNA at 65°C.

suppress the appearance of labeled adenovirus-specific RNA in polysomes if the same cellular mechanisms are involved in adenovirus mRNA processing. Late viral RNA was obtained from cells infected for 16 hr, treated with cordycepin (50 μg/ml) for 10 min, and then labeled for 45 min with [^{3}H]adenosine. Polysomes were isolated and the EDTA-released RNA was sedimented on sucrose gradients. Fig. 2 shows that cordycepin inhibited the accumulation of label in polysomal RNA late in the adenovirus infection by 85–90%. Under the same conditions, the inhibition does not exceed 70% in uninfected cells (7). The EDTA-released polysomal RNA was extracted with phenol, selected on adenovirus DNA, and then analyzed for poly(A) content. Fig. 2 shows that whereas poly(A) comprises about 10% of the total adenovirus-specific RNA in untreated control cells, it is absent from the adenovirus-specific polysomal RNA from cordycepin-treated cells.

DISCUSSION

The present study established that nuclear and polysomal adenovirus RNA, both early and late in the infectious cycle, contain an adenylate-rich segment, 150–250 nucleotides in length. As is true for uninfected cells, the percentage of poly(A) is much higher in the virus-specific polysomal RNA than in the virus-specific nuclear RNA. The poly(A) associated with adenovirus-specific RNA appears to correspond to the poly(A) recovered from the RNA of uninfected cells.

The poly(A) derived from adenovirus RNA appears not to be due to contaminating cellular sequences, since selection by adenovirus DNA gave at least 97% pure adenovirus polysomal RNA, and the percentage of poly(A) in these molecules was 5–10%. In addition, about 75% of the polysomal adenovirus-specific sequences were bound both to poly(U)–cellulose and in the Millipore-binding assay for molecules containing poly(A) (Tables 4 and 5).

The poly(A) in adenovirus-specific RNA appears obligatory for proper processing, transport, or translation of mRNA. Such a role has already been proposed for the poly(A) associated with the RNA of uninfected cells (6). This proposal is based on the results obtained with cordycepin, an analogue of adenosine. Whereas this drug has relatively little effect on HnRNA transcription, it severely inhibits the addition of poly(A) to both HnRNA and polysomal RNA (6), and at the same time decreases the accumulation of mRNA in the polysomes (7). The cordycepin inhibition of the labeling of polysomal RNA is, however, not complete, either in uninfected cells where it amounts to around 70% (7), or late in adenovirus infection where it amounts to 90% (Fig. 2). The small fraction of polysomal RNA remaining after cordycepin treatment of infected cells lacks poly(A) and may represent unique species of adenovirus mRNA. However, the nuclear leakage in late adenovirus infection is too extensive to safely conclude that this material is mRNA.

Adenovirus DNA replicates and is transcribed in the nucleus of infected cells (19, 20). The viral-specific RNA appears to be processed by cellular systems and transported to the cytoplasm, where it is translated in the polysomes. The adenovirus system offers many advantages as a model for the study of the possible processing of nuclear RNA into mRNA, because viral RNA sequences can be specifically identified by DNA–RNA hybridization. Detailed biochemical knowledge concerning the nuclear origin and processing of mRNA may, therefore, first become available from studies of virus-infected cells.

TABLE 5. *Adenovirus-specific sequences in poly(U)–cellulose fractions of polysomal RNA*

Elution temperature, °C	cpm Eluted	cpm Hybridized to adenovirus DNA*	% of Total adenovirus-specific cpm
4	460,000	109,900	43
25	300	—	—
50	170,000	124,000	57

[^{3}H]adenosine-labeled polysomal RNA from late after infection was passed through a column of poly(U)–cellulose. Bound and unbound fractions were assayed as in Table 4.

* After 48 hr of exposure to 80 μg of DNA at 65°C.

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