Proceedings of the National Academy of Sciences
Vol. 68, No. 3, pp. 557–560, March 1971

Biochemical Studies on Adenovirus Multiplication, XIX. Resolution of Late Viral RNA Species in the Nucleus and Cytoplasm

J. THOMAS PARSONS*, JACQUELINE GARDNER, AND MAURICE GREEN†

Saint Louis University School of Medicine, Institute for Molecular Virology, St. Louis, Missouri 63104

Communicated by Robert J. Huebner, December 28, 1970

ABSTRACT **Late after infection of cultured human cells (KB) with adenovirus type 2, the nucleus contains heterogeneous viral RNA species ranging in size from 10 to 43 S. Four viral RNA species found in the nucleus (36, 38, 40, and 43 S) are synthesized predominantly during a 15-min labeling period with [^{3}H]uridine, while smaller RNA species accumulate when labeling is continued for longer periods. In contrast, 6–8 viral RNA species, of sedimentation coefficient from 10 to 29 S, are found in the cytoplasm after a 30-min pulse label and a 2-hr chase. DNA-RNA hybridization-competition experiments demonstrate that viral RNA sequences present in nuclear 36–43S RNA are also present in cytoplasmic and polyribosomal RNA, suggesting that at least some of the cytoplasmic viral-specific RNA molecules are derived by cleavage of high molecular weight precursors from the nucleus.**

Cultured human cells infected with adenovirus type 2 (Ad 2) transcribe specific adenovirus gene sequences during productive infection (1–3). Before viral DNA synthesis (6 hr after infection) virus-specific RNA is transcribed from 15–20% of the Ad 2 genome (2); most of this RNA is detected as two major viral species, with sedimentation coefficients of 23S and 17S (4, 5). Late after infection (16–18 hr), virus-specific RNA is transcribed from 85 to 95% of the adenovirus genome (3). The virus-specific RNA species synthesized late after infection code for the synthesis of the 8–10 known capsid polypeptides, which range in molecular weight from 15,000 to 130,000 (6–9). We report here an analysis of the late-viral RNA species, present at 16–18 hr after infection, which reveals that high molecular weight viral RNA species sedimenting at 36–43 S are found in the nucleus and appear to be precursors of the 10–29S viral-specific RNA species found in the cytoplasm.

MATERIALS AND METHODS

Infection, cell fractionation, and RNA isolation

Suspension cultures of KB cells, at 2–3 × 10^5 cells/ml, were infected with Ad 2 (strain 38-2) at an input multiplicity of 50 plaque-forming units (PFU)/cell, as described previously (3). [^{3}H]uridine (4 μCi/ml, 20 Ci/mmol) was added to infected suspension cultures, cells were harvested at various times, and nuclear and cytoplasmic RNA were isolated by sodium dodecyl sulfate–phenol extraction (4).

DNA-RNA hybridization and hybridization-competition

These measurements were performed with viral DNA immobilized on nitrocellulose filters as described (10, 11). Ad 2 DNA was prepared from highly purified virus (12).

Polyacrylamide gel electrophoresis and zone centrifugation in sucrose density gradients

Labeled RNA from infected cells was resolved on either 2.8 or 3.1% polyacrylamide gels, the gels were sliced, the RNA was eluted from each slice, and the viral RNA was quantitated by hybridization with viral DNA (4).

RESULTS

Ad 2 late RNA species in the cell nucleus

Nuclear RNA species from KB cells labeled with [^{3}H]uridine for 15 min and 60 min, at 18 hr after infection with Ad 2, were resolved by electrophoresis on 2.8% polyacrylamide gels. Radioactive RNA was eluted from the gel slices and annealed with Ad 2 DNA. As many as 10 virus-specific RNA species larger than 26 S can be detected after 15- and 60-min labeling periods (Fig. 1). N_I–N_{IV} appear to be the major viral RNA species labeled during a 15-min pulse with [^{3}H]uridine (Fig. 1*A*). Increasing proportions of N_{VI}–N_X, as well as at least three smaller RNA species, were evident after 60 min of labeling (Fig. 1*B*). Treatment of nuclear RNA, labeled for 60 min, with 8 M urea (Fig. 2) or 95% dimethylsulfoxide (not shown) did not alter the number of RNA species nor their rate of migration. Since it has been demonstrated that such treatment is sufficient to disrupt much of the secondary structure of the 70S RNA of murine leukemia virus (13, 14), we conclude that these large viral RNA species in the nucleus are intact adenovirus RNA molecules with molecular weights as high as 4 × 10^6 (Table 1), and do not represent RNA species aggregated during isolation of the nuclear RNA. We cannot rule out completely the possibility that N_I–N_{IV} represent one or two virus-specific RNA molecules at different stages of cleavage. However, all four RNA species were detected in about the same relative amounts after a brief (7 min) labeling period, suggesting that they probably represent discrete nucleotide sequences.

Abbreviation: Ad 2, adenovirus type 2.

* Postdoctoral fellow (PF-500) of the American Cancer Society. Present address: Institut fur molekularbiologie, Universitat Zurich, Zurich, Switzerland.

† Research Career Awardee (5-K6-AI-4739), National Institutes of Health, U.S. Public Health Service.

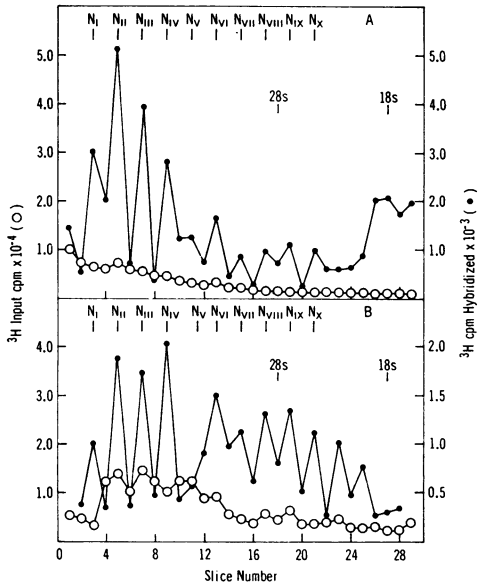

FIG. 1. Polyacrylamide gel electrophoresis of late Ad 2 RNA from the nucleus. KB cells, infected for 18 hr, were labeled with [^{3}H]uridine (4 μCi/ml) for 15 min (*A*) and 60 min (*B*). Nuclear RNA was isolated from purified nuclear preparations as described (4), 50–75 μg of [^{3}H]RNA was applied to 2.8% polyacrylamide gels (8 cm), and electrophoresis was for 6 hr at 5 mA per tube. The gels were sliced; each slice was solubilized and hybridized. The position of 18S and 28S rRNA (ribosomal RNA) was determined by parallel electrophoresis of rRNA under identical conditions. Input ^{3}H cpm per slice (O); ^{3}H cpm hybridized to 1 μg of Ad 2 DNA (●).

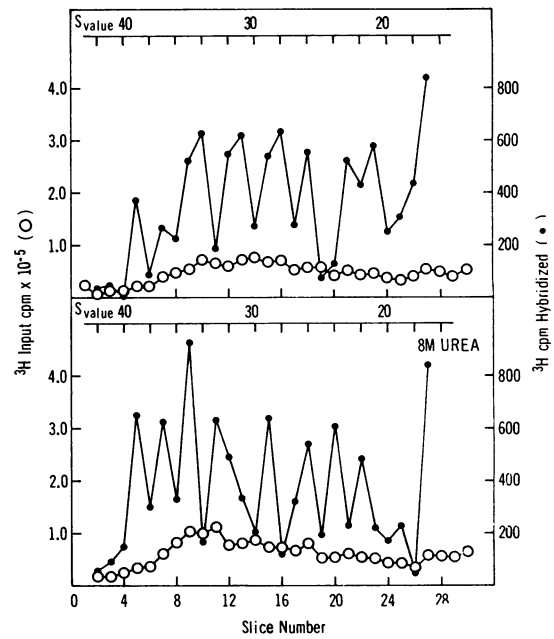

FIG. 2. Polyacrylamide gel electrophoresis of urea-treated, late Ad 2 RNA from the nucleus. Nuclear RNA was isolated from cells infected for 18 hr and labeled with [^{3}H]uridine for 60 min. 50 μg of untreated [^{3}H]RNA (*top*) and 50 μg of denatured [^{3}H]-RNA (dissolved in 8 M urea–10 mM TES (Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid–2 × SSC*, 30 min at 37°C) were applied to polyacrylamide gels and electrophoresed for 6 hr at 5 mA per tube. The distribution of viral RNA species was determined as described in the legend to Fig. 1. Input ^{3}H cpm per slice (O); ^{3}H cpm hybridized to 1 μg of Ad 2 DNA.

* 2 × SSC, 0.30 M NaCl–0.030 M sodium citrate.

Ad 2 late RNA species in the cytoplasm

Cytoplasmic RNA was isolated from infected cells labeled with [^{3}H]uridine from 16 to 18 hr after infection, the RNA was resolved by electrophoresis on 3.1% polyacrylamide gels for 3 hr, and virus-specific RNA was identified by annealing with Ad 2 DNA (Fig. 3). 8–10 virus-specific RNA species between 12 and 36 S were resolved. The rate of migration of the viral RNA species remained the same after treatment with either 8 M urea or 95% dimethylsulfoxide. To determine whether the 30–36S RNA present in the cytoplasm represented a stable viral-specific RNA population, infected cells were pulse labeled for 30 min with [^{3}H]uridine at 16 hr after infection, then chased by incubation with an excess of unlabeled uridine for 2 hr at 37°C. Less than 10% of the labeled RNA remained in the nucleus. Cytoplasmic RNA was isolated and resolved by electrophoresis on 3.1% polyacrylamide gels for 3 hr (Fig. 4*A*) or 5 hr (Fig. 4*B*). In contrast to the continuous-labeling experiment, no virus-specific RNA was detected with a sedimentation coefficient larger than 30 S. 6–8 virus-specific RNA species between 12 and 30 S were resolved after short electrophoresis (Fig. 4*A*) and these were further separated by longer electrophoresis (Fig. 4*B*). The viral RNA species present after a pulse–chase experiment (L_I–L_{VIII}) probably represent stable, functional viral mRNA molecules transcribed late after infection. As shown in Table 2, there is good correlation between the estimated polypeptide equivalent of several of the viral RNA species and a specific adenovirus structural polypeptide (6–9), e.g., L_{II} may code for hexon, L_{IV} for penton, L_V for fiber, and L_{VI} for core.

TABLE 1. *Nuclear adenovirus RNA species synthesized late after infection*

	Sedimentation coefficient*	Molecular weight†	% Adenovirus genome‡
N_I	43S	4.3×10^6	38
N_{II}	41S	3.8×10^6	33
N_{III}	38S	3.2×10^6	28
N_{IV}	36S	2.7×10^6	24
N_V	34S	2.5×10^6	22
N_{VI}	32S	2.2×10^6	19
N_{VII}	30S	2.0×10^6	18
N_{VIII}	29S	1.8×10^6	16
N_{IX}	27S	1.6×10^6	14
N_X	26S	1.4×10^6	12

* Sedimentation coefficients were determined by the rate of migration of the RNA species in 2.8% polyacrylamide gels relative to that of 28S and 18S rRNA.

† Calculated from molecular weight = $1550\ S^{2.1}$ (15).

‡ Ad 2 DNA has a molecular weight of 23×10^6.

Are high molecular weight nuclear RNA species precursors to cytoplasmic viral RNAs?

The extent of sequence homology between the high molecular weight nuclear RNA and cytoplasmic RNA was determined by DNA–RNA hybridization–competition experiments. Labeled nuclear RNA was isolated by centrifugation in sucrose density gradients; those fractions sedimenting at

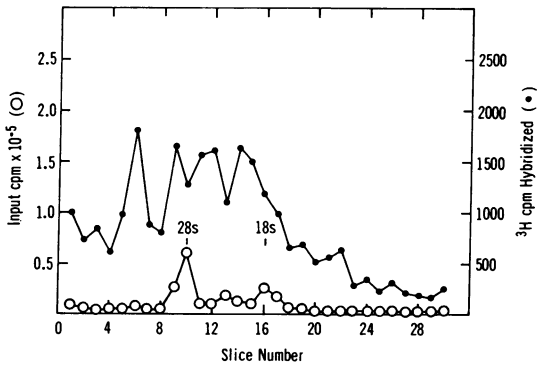

FIG. 3. Resolution of late, cytoplasmic Ad 2 RNA. KB cells infected for 16 hr were labeled for 2 hr with 4 μCi/ml of [³H]-uridine. RNA was purified from the cytoplasm (4) and 100–125 μg of [³H]cytoplasmic RNA was applied to 8-cm 2.8% polyacrylamide gels. Electrophoresis was for 3 hr at 5 mA/tube. The gels were sliced, and the RNA was eluted from each slice and hybridized to 1 μg of Ad 2 DNA. The position of 28S and 18S RNA was determined by coelectrophoresis of [¹⁴C]rRNA. Input ³H and ¹⁴C cpm per slice (O); ³H cpm hybridized to 1 μg of Ad 2 DNA.

greater than 35 S were pooled. This high molecular weight nuclear RNA readily saturated 0.1 μg of Ad 2 DNA (Fig. 5, *inset*). In a two-step hybridization–competition experiment, increasing amounts of late cytoplasmic RNA, late polyribosomal RNA, and early RNA (6 hr after infection) were annealed with 0.1 μg of Ad 2 DNA for 24 hr. Saturating

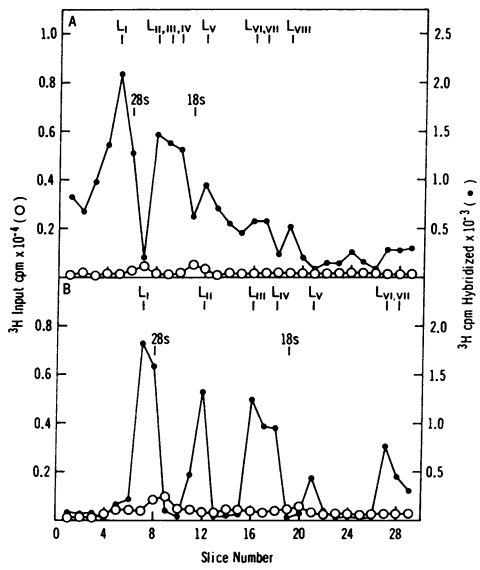

FIG. 4. Resolution of late cytoplasmic, pulse–chase, Ad 2 RNA. KB cells infected for 17 hr were labeled for 30 min with 4 μCi/ml of [³H]uridine. The cells were then transferred to fresh medium containing unlabeled uridine and the cells were incubated for 2 hr. RNA was purified from the cytoplasm (4) and 100–125 μg of [³H]cytoplasmic RNA was applied to 8-cm 3.1% polyacrylamide gels. Electrophoresis was for 3 hr (*A*) and 5 hr (*B*). The gels were sliced; the RNA was eluted from each slice and hybridized to 1 μg of Ad 2 DNA. The position of 28S and 18S RNA was determined by parallel electrophoresis of [³H]rRNA. Input ³H cpm per slice (O); ³H cpm hybridized to 1 μg of Ad 2 DNA.

TABLE 2. *Cytoplasmic adenovirus RNA species synthesized late after infection*

	Sedimentation coefficient*	Estimated molecular weight†	Polypeptide equivalent	Adenovirus capsid peptides‡
L_I	29S	1.82×10^6	200,000	
L_{II}	24S	1.23×10^6	136,000	Hexon—130,000
L_{III}	21S	0.93×10^6	103,000	
L_{IV}	19S	0.74×10^6	82,000	Penton—70,000
L_V	16S	0.56×10^6	62,000	Fiber—62,000
L_{VI}	14S	0.40×10^6	44,000	Core—44,000
L_{VII}	12S	0.29×10^6	32,000	
L_{VIII}	10S	0.19×10^6	21,000	Core—24,000

* Sedimentation coefficients were determined by the rate of migration of the RNA species in polyacrylamide gels relative to 18S and 28S RNA.

† Calculated from molecular weight = $1550\ S^{2.1}$ (15).

‡ Molecular weights of the capsid proteins were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (6–9).

amounts of labeled high molecular weight nuclear RNA was then added, and a second annealing reaction was performed for an additional 24 hr. Cytoplasmic and polyribosomal RNA both competed effectively with this nuclear RNA for sites on viral DNA. We conclude that all high molecular weight nuclear RNA sequences of viral-infected cells are present in

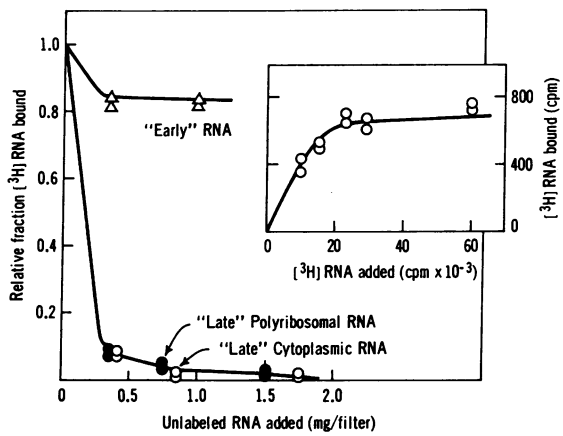

FIG. 5. Hybridization–competition between late, high molecular weight nuclear [³H]RNA and unlabeled early, late polyribosomal, and late cytoplasmic RNA. 200–300 μg of late, nuclear [³H]RNA (labeled 60 min with [³H]uridine) was layered on a 10–30% sucrose gradient (containing 0.15 M NaCl–0.015 M sodium citrate (SSC)–10 mM EDTA, pH 7.2) and centrifuged for 4.5 hr at 36,000 rpm. Nuclear [³H]RNA fractions sedimenting faster than 35 S were pooled and concentrated by ethanol precipitation. Membrane filters containing 0.1 μg of Ad 2 DNA were annealed for 20 hr at 66°C in 2 × SSC with increasing amounts of nuclear [³H]RNA (*inset*). To carry out the hybridization–competition experiment, membrane filters containing 0.1 μg of Ad 2 DNA were first incubated with increasing amounts of early RNA, late polyribosomal RNA, and late cytoplasmic RNA. After 24 hr at 66°C, each filter was further incubated for 20 hr at 66°C with 3.0×10^4 cpm of nuclear [³H]RNA. The filters were washed, treated with RNase, washed, and counted as described (10, 11).

cytoplasmic and polyribosomal RNA, and probably represent precursors to at least some of the cytoplasmic viral RNAs.

DISCUSSION

Virus-specific RNA sequences transcribed late after infection with Ad 2 appear to be transcribed as large precursor RNA molecules within the cell nucleus and to be subsequently cleaved to smaller RNA species prior to translation in the cytoplasm. This conclusion is supported by the following: (*1*) at least four Ad 2 RNA species with sedimentation coefficients of 36–43 S are rapidly synthesized in the nucleus but do not appear as 36–43 S viral RNA species within the cytoplasm after a pulse–chase period. (*2*) The size distribution of the 36–43S nuclear RNA is not altered by treatment with 8 M urea or 95% dimethylsulfoxide, conditions that are known to alter the size distribution of murine leukemia virus RNA, and (*3*) both total cytoplasmic RNA and polyribosomal RNA block the binding of high molecular weight [^{3}H]RNA from the nucleus to Ad 2 DNA.

Our data present evidence that late gene expression is controlled at least at two levels: (*1*) transcriptional and (*2*) post-transcriptional. (*1*) *transcription* of virus-specific RNA synthesis is controlled by specific binding of RNA polymerase to a limited number of initiation regions, resulting in the transcription of large precursor viral RNA molecules. For example, assuming that the nuclear RNAs N_I, N_{II}, and N_{III} are discrete nucleotide sequences, the transcription of these three RNA species would result in the transcription of as much as 95% of the Ad 2 DNA. (*2*) The *post-transcriptional* control occurs during the cleavage of precursor viral RNA molecules from the nucleus to smaller, virus-specific RNA prior to translation on the cytoplasmic polyribosomes. The heterogeniety in size distribution of the virus-specific RNA of the nucleus probably reflects the cleavage of these precursor RNAs to smaller RNA species within the nucleus. While the detection of 30–36S cytoplasmic viral RNA in continuous-labeling experiments, but not in pulse-chase experiments, suggests that some precursor RNA molecules may be cleaved within the cytoplasm, we cannot readily distinguish between viral RNA species undergoing cleavage in the cytoplasm and viral RNA that appears in the cytoplasm as a result of leakage from the nucleus *in vivo* or *in vitro*.

The calculated size of the polypeptides coded for by L_{II}, L_{IV}, L_V, L_{VI} and the known structural polypeptides of the adenovirus virions (Table 2) are compatible with the specific cleavage of precursor RNAs to monocistronic mRNAs. The observation of L_I, which could code for a polypeptide chain of 180–200,000 daltons, and the absence of any polypeptide chain that large in infected cells (9), suggests that large polypeptides may be synthesized and specifically cleaved to smaller peptides, as has been recently shown for poliovirus proteins (16) and for the core peptides of vaccinia virus (17).

While the transcription of Ad 2 DNA and the post-transcriptional cleavage of Ad 2 RNA appear similar to that observed for herpes virus DNA (18) and SV40 DNA (19), it appears uniquely different from the transcription and cleavage of ribosomal RNA (20–23) and nuclear DNA-like RNA (24–28). The processing of nucleolar rRNA and nuclear DNA-like RNA results in the specific cleavage and degradation of a portion of the initial RNA sequence. In contrast, all the nucleotide sequences contained in the 36–43S Ad 2 nuclear RNA are present in polyribosomal RNA, demonstrating that these RNA sequences are conserved during post-transcriptional cleavage and transport to the cytoplasm. The experiments presented here provide techniques to identify specific precursor viral RNAs within the cell nucleus and to follow the fate of these RNA species within the cell and, in addition, present an unique opportunity to study in detail the mechanism that governs the processing, transport, and translation of a defined viral mRNA molecule.

The authors wish to thank Drs. David Schlessinger and Heschel Raskas for their helpful advice in the preparation of this manuscript.

This investigation was supported by USPHS grant AI-01725 and research contract PH43-64-928 from the National Institute of Allergy and Infectious Diseases, Infectious Disease Branch, National Institutes of Health, and research contract PH43-67-692 from the National Cancer Institute, Viral Carcinogenesis Branch, Etiology Area, National Institutes of Health, USPHS, Bethesda, Md.

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