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Fidelity of Adenovirus RNA Transcription in Isolated HeLa Cell Nuclei

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An in vitro nuclear system from adenovirus type 2-infected cells was developed to study transcription of viral RNA. Nuclei isolated from adenovirus-infected HeLa cells late in the infectious cycle synthesized in vitro only RNA from the r-strand of adenovirus DNA. Around 15% of the virus-specific RNA in isolated nuclei was polyadenylated. Short pulse labeling of nascent RNA followed by hybridization of size-fractionated RNA to specific restriction endonuclease fragments of the genome suggested that the origin(s) for transcription is located on the r-strand in the left 30% of the adenovirus 2 genome at late times in the infectious cycle. Pulse-chase experiments were used to estimate the elongation rate of adenovirus high-molecular-weight RNA in isolated nuclei. An elongation of at least six nucleotides per second was observed in vitro. Viral RNA synthesis in in vitro nuclei showed several similarities to the in vivo system late in the infectious cycle.

Studies on transcription and post-transcriptional modification have been simplified by the introduction of nuclear in vitro systems. Specific labeling of the precursor triphosphate nucleotides (23), reduction of pool sizes allowing pulse-chase experiments (13), and the use of selective inhibitors that normally do not penetrate the plasma membrane are the most noticeable advantages. The fungal toxin α-amanitin has been exceptionally useful in identifying the different RNA polymerases of eukaryotic cells since the polymerases exhibit differential sensitivities to α-amanitin. RNA polymerase I, which is insensitive to α-amanitin, has been shown to transcribe the ribosomal precursor RNA in isolated rat liver nuclei (2) and in HeLa cell nuclei (35). HeLa cell heterogeneous nuclear RNA and simian virus 40 and adenovirus high-molecular-weight RNA are transcribed by RNA polymerase II, which is inactivated at low concentrations of the fungal toxin (8, 20, 31, 35). RNA polymerase III, which has intermediate sensitivity, synthesizes low-molecular-weight RNA like 5S RNA (20), the precursors to tRNA (15), and the adenovirus-associated (VA) RNA (20, 27, 32).

Polyadenylation of RNA synthesized in isolated nuclei has been observed (14, 22) and has been shown to be dependent on high concentrations of ATP and cytoplasmic extracts in HeLa cell nuclei (9).

Fidelity of transcription in isolated nuclei has been demonstrated for both RNA polymerases I and III. rRNA is transcribed from one of the DNA strands in *Xenopus laevis* nuclei, and the spacer regions are probably not transcribed (24). Precursor tRNA and 5S RNA are transcribed from the correct strand in mouse myeloma cell nuclei (15).

Specific processing of ribosomal precursor RNA molecules into lower-molecular-weight RNA of discrete sizes occurs in isolated mouse myeloma nuclei (14) and in HeLa nuclei (3). Precursor tRNA is also cleaved to the 4S product in isolated mouse myeloma nuclei (15). Raskas (21) studied the processing of heterogeneous nuclear RNA in isolated nuclei from adenovirus-infected cells. High-molecular-weight virus-specific RNA was broken down to lower-molecular-weight RNA and transported from the nucleus to the incubation medium. The specificity of the processing was, however, not analyzed.

The present investigation examines the fidelity of adenovirus RNA synthesis in isolated HeLa cell nuclei isolated late in the infectious cycle. Transcription of high-molecular-weight RNA occurs only from the r-strand of adenovirus type 2 (Ad2) DNA. Short pulse-labeling experiments show that the short nascent RNA originates from the left-hand end of the Ad2 genome and that the nascent RNA increases in size as the right-hand end is approached. This implies a common origin(s) for adenovirus heterogeneous nuclear RNA synthesis in the left end of the genome, in agreement with results

obtained in vivo (1). Pulse-chase labeling in the isolated nuclei indicate that processing of high-molecular-weight RNA to defined species of lower molecular weight is negligible at late times in the infectious cycle, but turnover of the in vitro-synthesized RNA can be observed.

MATERIALS AND METHODS

Cells and virus infection. HeLa cells were grown in Eagle spinner medium at a cell density of 10^5 to 5 $\times$ 10^5/ml. The cells were infected with Ad2 at a multiplicity of 5,000 particles/cell for 30 min at a cell concentration of 10^7 cells/ml. The cells were then diluted to 3 $\times$ 10^5 cells/ml and harvested at 14 to 16 h postinfection by adding frozen phosphate-buffered saline (12).

Cell fractionation. The cells were washed twice in buffer A, containing 125 mM KCl, 30 mM Tris-hydrochloride, pH 7.4, 5 mM $Mg(Ac)_2$, and 1 mM dithiothreitol, and then allowed to swell in 10 mM KCl, 30 mM Tris-hydrochloride, pH 7.4, 5 mM $Mg(Ac)_2$, and 1 mM dithiothreitol for 10 min. Nuclei were prepared by homogenization in a Dounce homogenizer with a B-pestle. The homogenate was layered on a cushion of incubation buffer (buffer A with 25% glycerol) and centrifuged at 400 $\times$ *g* for 5 min. The nuclei were resuspended in incubation buffer at a density of 2 $\times$ 10^7 nuclei/ml. The purity of the nuclei was checked by phase-contrast microscopy.

RNA transcription in isolated nuclei. Nuclei were incubated in incubation buffer at 30°C after addition of ATP to 1 mM and CTP, GTP, and UTP to 0.25 mM. When CTP, UTP, or GTP was used as label, the concentration of the ^{3}H-labeled nucleotide was lowered to 0.025 mM and it was added at a specific activity of 0.8 Ci/mmol (Amersham Radiochemical Centre). When labeling with [^{3}H]ATP, the concentration was kept at 0.1 to 0.2 mM, with a specific activity of 3.5 Ci/mmol.

Extraction of RNA. The incubation mixtures were phenol extracted as described by Holmes and Bonner (7). After ethanol precipitation, the nucleic acids were resuspended in 10 mM NaCl, 10 mM Tris-hydrochloride, pH 7.8, and 10 mM $MgCl_2$ and digested with DNase at 100 μg/ml (RNase-free Worthington DPFF, further purified by gel filtration on Sephadex G-100). The RNA was phenol extracted and again precipitated with ethanol. Oligo(dT)-cellulose chromatography of RNA was performed as previously described (29). The fraction which percolated the column is called poly(A)− RNA, and the retained fraction is called poly(A)+ RNA.

Hybridization. Hybridization was carried out with Ad2 DNA on nitrocellulose filters in 500 mM NaCl, 50 mM Tris-hydrochloride, pH 7.8, 1 mM EDTA, 0.5% sodium dodecyl sulfate in 50% formamide for 24 to 48 h. After hybridization, the filters were washed repeatedly with 2× SSC (SSC = 0.15 M NaCl + 0.015 M sodium citrate) and treated with 20 μg of RNase A per ml for 30 min at 37°C. Formamide was purified according to the method of Tibbetts et al. (28).

Separation of the complementary strands of Ad2 DNA was performed by poly(U,G) binding followed by CsCl centrifugation (29). The strand with low density corresponds to the strand that is transcribed in the rightward direction (the r-strand), and the heavy strand corresponds to the leftward transcription (the l-strand) (5). Liquid hybridization of labeled RNA to cold adenovirus DNA strands involving analysis of the RNase-treated hybrids on Sephadex G-100 has also been described (17). Exhaustive hybridization of adenovirus RNA synthesized in isolated nuclei was carried out with DNA on filters as previously described (12). Under these conditions, only products formed from large segments of the genome will be exhaustively hybridized in DNA excess. Products formed in large amounts from genes where reinitiation occurs will not hybridize quantitatively due to low specific activity. Thus in the present experiments polymerase II products will probably be exhaustively hybridized, but polymerase III products cannot be scored quantitatively unless the DNA concentration is increased 10- to 100-fold, which is prohibitive. Cold VA RNA (VAI and VAII) was, however, added in 200-fold excess over the concentration of VA DNA on the filters in some experiments to rule out hybridization of labeled VA RNA.

Sucrose gradient analysis. Zonal rate sedimentation was performed at 25°C in 15 to 30% sucrose gradients. For analysis under nondenaturing conditions, the gradients contained 100 mM NaCl, 10 mM Tris-hydrochloride, pH 7.8, 1 mM EDTA, and 0.5% Sarkosyl. Denaturing gradients in 70% formamide contained 50 mM Tris-hydrochloride, pH 7.8, 2 mM EDTA, and 0.1% sodium dodecyl sulfate, and the samples were first denatured in 90% formamide at 50°C in the same buffer. It was separately ascertained that double-stranded reovirus RNA sedimented in the denatured form on these gradients. Gradients were centrifuged at 40,000 rpm in the Spinco SW40 rotor for times indicated in the figure legends. Adenovirus-specific RNA in gradient fractions was hybridized to Ad2 DNA on nitrocellulose filters (17).

Poly(A) analysis. For analysis of poly(A) tracts in viral RNA, [^{3}H]ATP-labeled RNA was hybridized to Ad2 DNA on nitrocellulose filters. The filters were washed, and the poly(A) from the selected RNA was released by incubating filters with 5 μg of RNase A and 20 U of RNase T1 per ml in 200 mM NaCl, 10 mM Tris-hydrochloride, pH 7.8, and 1 mM EDTA for 30 min at 37°C. The released poly(A) tracts were phenol extracted and analyzed, after ethanol precipitation, on 15% polyacrylamide gels (12).

Isolation of restriction enzyme fragments of Ad2 DNA. Endonucleases endo R·*Bam*HI and *Eco*RI were purified and cleavages were carried out as described previously (17, 27). DNA fragments were separated by electrophoresis on 0.7% agarose gels in 40 mM Tris-acetate (pH 8.3) with 1 mM EDTA and 5 mM sodium acetate. The gels were stained with ethidium bromide, and fragments were eluted by dissolving gel slices in 5 M $NaClO_4$. The DNA was then adsorbed to hydroxylapatite, washed, and eluted with 0.5 M phosphate buffer, pH 7.5, as de-

scribed previously (11). After dialysis, the DNA was fixed to nitrocellulose filters as previously described (12).

RESULTS

Optimal RNA synthesis in in vitro nuclei. Prolonged transcription in isolated nuclei and extensive polyadenylation of the synthesized RNA appears to require cytoplasmic extracts and high concentrations of ATP in the incubation mixture (9, 34). There might not be an absolute requirement for cytoplasmic extracts, since polyadenylation has also been observed in a chromatin transcription complex (19). In a viral transcription system it would be preferable to omit cytoplasmic extracts in order to obtain a high specific activity of synthesized RNA, which would facilitate hybridization analysis of the products. The requirement for cytoplasmic extracts and nucleotide composition for synthesis of RNA in isolated nuclei from adenovirus-infected cells was first investigated. RNA synthesis in isolated nuclei continued for more than 1 h, irrespective of whether cytoplasm was included or omitted (Fig. 1). Nuclei incubated with desalted cytoplasm showed only a slightly higher rate of RNA synthesis than nuclei incubated in buffer or with an unfractionated cytoplasm. The difference in rate between the two cytoplasmic extracts was probably due to a higher specific radioactivity in the nucleoside triphosphate pool with the desalted extract. RNA synthesis in cytoplasmic extract alone was almost negligible. About the same rate of transcription was observed when the nucleotide triphosphate concentration was varied between 0.2 and 1 mM for UTP, GTP, and CTP. There appeared to be a requirement for ATP, since a significantly lower rate was observed below 1 mM ATP. In the following experiments we used an incubation mixture without cytoplasmic extract with the composition described in Material and Methods. The initial rate of transcription for the nuclei in this system was calculated to about 20,000 nucleotides (n) per s per nucleus. The rate dropped to less than 7,000 n/s after 1 h of incubation.

Transcription and polyadenylation of adenovirus RNA. To investigate the extent of viral transcription and the polyadenylation of viral RNA, nuclei were incubated with 100 μM [^{3}H]ATP under otherwise standard conditions. RNA was extracted from the nuclei and fractionated into poly(A)− and poly(A)+ RNA by oligo(dT)-cellulose chromatography. Table 1 shows that 15% of the labeled RNA was polyadenylated in vitro. Analysis of viral versus cellular transcription by exhaustive hybridization

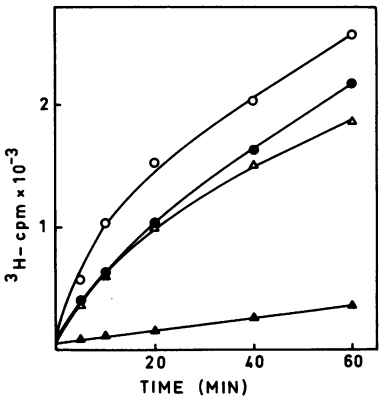

FIG. 1. *RNA synthesis in nuclei isolated at 16 h postinfection from adenovirus-infected cells. Nuclei were incubated at a concentration of 2 × 10^7 nuclei/ml with buffer as described in Materials and Methods (●). Cytoplasmic extracts corresponding to 2 × 10^7 cell equivalents/ml were added to the nuclei (△). Addition of the same amount of cytoplasmic extract passed through a Sephadex G-25 column (○). RNA synthesis in cytoplasmic extract without the addition of nuclei (▲). [^{3}H]CTP was used as label.*

TABLE 1. *Polyadenylation of adenovirus RNA in isolated nuclei*[a]

RNA	Radioactivity (cpm × 10^{-3})	Percent of input	Hybridized to Ad2 DNA (cpm × 10^{-3})	Percent of viral origin
Poly(A)−	191	83	105	55
Poly(A)+	33.9	15	19.7	58

[a] Nuclei isolated at 14 h postinfection were incubated with 100 μM [^{3}H]ATP as label for 45 min at 30°C. The RNA was subjected to oligo(dT)-cellulose chromatography. Hybridization was carried out to 20 μg of Ad2 DNA on filters.

to viral DNA on filters revealed that 55% of poly(A)− RNA and 58% of poly(A)+ RNA was of viral origin, in agreement with the results obtained under in vivo conditions (18). To measure the length of the poly(A) moiety from in vitro nuclei, viral RNA was isolated in a separate experiment by hybridization to adenovirus DNA on filters; the hybridized RNA was RNase treated in a high-salt buffer, and the released poly(A) was analyzed on 15% polyacrylamide gels as described in Material and Methods. In vitro, nuclei incorporated [^{3}H]adenosine in a 200-nucleotide poly(A) segment of viral RNA (Fig. 2). Five percent of the label in viral RNA was found in poly(A), which suggests a mean molecular weight of 1.2 × 10^6 for the viral RNA,

assuming 25% A-residues in the internal region of the mRNA. These results suggest that most if not all of the poly(A) had been polymerized in vitro.

There was also a low-molecular-weight RNase-resistant material (Fig. 2), which may correspond to an oligo(A) stretch or, alternatively, to RNA sequences that are unspecifically released from the hybridization filters. This material will be studied separately.

Sedimentation analysis in sucrose gradients under nondenaturing conditions of RNA synthesized in isolated nuclei showed two populations of RNA (Fig. 3.) The molecular weight of the fast-sedimentating RNA ranged from 1×10^6 to 6.5×10^6, with an average of 2.5×10^6. The low-molecular-weight RNA, consisting mostly of VA RNAs and precursor tRNA (27), comprised about 25% of the total RNA.

When oligo(dT)-fractionated RNA was analyzed in formamide gradients under denaturing conditions (Fig. 4), both the poly(A)+ and poly(A)− RNA sedimented heterogeneously, with molecular weights ranging from 5×10^4 to 3×10^6. This size range is significantly lower than that observed after sedimentation analysis under nondenaturing conditions (Fig. 3), but in reasonable agreement with the average molecular weight estimate of 1.2×10^6 made from the poly(A) segment analysis (Fig. 2).

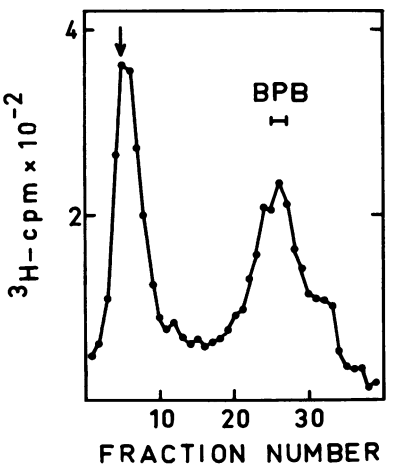

FIG. 2. *Polyacrylamide electrophoresis of poly(A) isolated from viral RNA. Viral RNA was labeled with [^{3}H]ATP and isolated by phenol extraction and hybridized to Ad2 DNA on filters. The filters were RNase treated in a high-salt buffer, and the released material was phenol extracted and analyzed on 15% polyacrylamide gels. The markers were run in a parallel gel, and the arrow corresponds to the position of poly(A) from adenovirus mRNA synthesized in vivo. BPB, Bromophenol blue dye marker.*

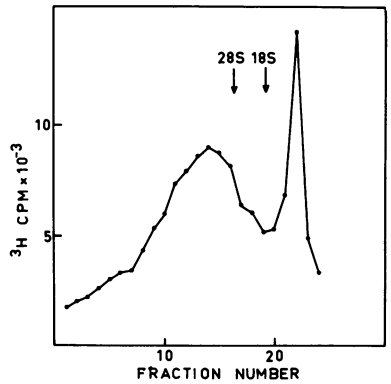

FIG. 3. *Nondenaturing sucrose gradient analysis of RNA synthesized in isolated nuclei. RNA was labeled for 45 min with [^{3}H]GTP, phenol extracted, and sedimented in 15 to 30% sucrose gradients for 3 h at 40,000 rpm in the Spinco SW40 rotor. The 28S and 18S markers from rRNA were analyzed in parallel gradients.*

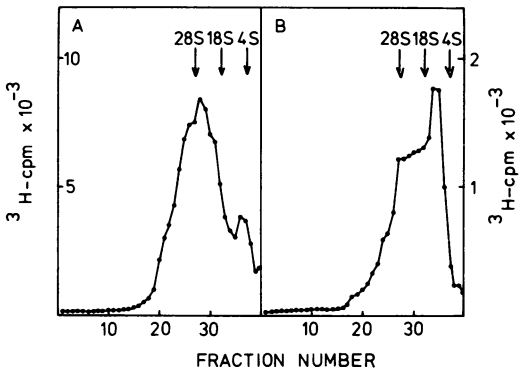

FIG. 4. *Formamide sucrose gradient analysis of poly(A)+ and poly(A)− RNA synthesized in isolated nuclei from adenovirus-infected cells. The incubation mixture contained [^{3}H]GTP as label, and the RNA was phenol extracted and fractionated on oligo(dT)-cellulose. The RNA was precipitated with ethanol and dissolved in 10 mM Tris-hydrochloride, pH 7.8. Formamide was added to 90%, and the samples were incubated at 37° for 5 min. The sample was diluted to 70% formamide and layered on 15 to 30% sucrose gradients prepared in 70% formamide in 0.05 M Tris-hydrochloride, pH 7.8, with 2 mM EDTA and 0.1% sodium dodecyl sulfate. Centrifugation was performed at 25°C for 16 h at 40,000 rpm in the Spinco SW40 rotor. (A) PolyA+ RNA; (B) PolyA− RNA.*

It may therefore be concluded that the transcripts in vitro appear to be comparatively large when analyzed on nondenaturing gradients or by the size of the poly(A)+ fragments. Similar results have been reported for the in vivo synthesis of nuclear RNA in adenovirus-transformed cells (6).

Specificity of transcription in isolated nuclei. Late in the adenovirus productive cycle, newly synthesized mRNA is preferentially derived only from the r-strand of the viral genome (10, 17). To establish whether this reflects a transcription control, isolated nuclei were labeled with [^{3}H]GTP for 50 min. The synthesized RNA was isolated from the nuclei and hybridized in liquid to the separated strands of Ad2 DNA. After hybridization and digestion with RNase, the samples were chromatographed on Sephadex G-100. The fraction of radioactivity eluting as DNA-RNA hybrid or duplex RNA in the void volume was determined. When RNA was self-annealed under hybridization conditions, 1.2% of the label eluted as duplex RNA (Table 2). Less than 0.05% of the RNA hybridized to the DNA l-strand, whereas 26% hybridized to the r-strand. The relative amount of self-annealed RNA in in vitro nuclei is comparable with the fraction of double-stranded viral RNA in adenovirus-infected cells at late times in the infectious cycle (17). Assuming that half of the duplex RNA is of l-strand origin, a maximum of 0.6% of the in vitro-labeled RNA could be l-strand specific, but since very little if any RNA hybridized to an excess of l-strand, the value is probably lower. Taken together, the results indicate a 40-fold or higher preference for r-strand transcription in isolated nuclei, which again is in total agreement with the in vivo conditions, where there is a 100-fold preference for the r-strand when newly synthesized mRNA is analyzed (17).

Size of the initial transcripts of r-strand-specific RNA. The adenovirus RNA is transcribed as a high-molecular-weight RNA, presumably starting at a common origin (1, 16, 30). Since the r-strand is predominantly transcribed, it is possible to carry out a Dintzis-type of experiment (4), which has already been performed under in vivo conditions (1). Thus, to confirm that there is one common origin(s) of viral transcription in isolated nuclei and an increasing molecular weight of nascent RNA the further the polymerase migrates from the origin along the r-strand, nuclei were pulse labeled with [^{3}H]UTP for 3 min. This is definitely within the synthesis time of a full transcript in isolated nuclei. The RNA was extracted and sedimented in sucrose gradients under nondenaturing conditions. All fractions were hybridized to 1-μg Ad2 DNA equivalents of restriction enzyme fragments *Bam*HI-B, -D, and -C followed by *Eco*RI-B and -D (25). The *Bam*HI fragments B, D, and C are in fact followed by the *Eco*RI fragments B, F, D, E, and C on the Ad2 DNA map, but only *Eco*RI fragments B and D are shown in the figure. Only small molecules, on the average 1.0 kilobases (kb) long, hybridized to *Bam*HI fragment B (Fig. 5). RNA complementary to *Bam*HI fragment D sediment as molecules that were 7.0 kb long. *Bam*HI fragment C-specific RNA was still larger, about 14 kb. The hybridization patterns show increasingly larger molecules as the right-hand end of the Ad2 genome is approached. The largest molecules, 20 to 30 kb, hybridized predominantly to the right-hand end of the genome. Experiments with *Eco*RI fragments F, E, and C showed that the RNA hybridizable to these fragments also sedimented in the range of 20 to 30 kb in the gradients (compare also with Fig. 7, where the terminal *Bam*HI fragment A was analyzed). A small fraction of low-molecular-weight RNA hybridized to all fragments.

In conclusion, the small nascent RNA hybridized predominantly to *Bam*HI fragment B, and the increasing molecular weight of the RNA correlated well with the distance from the left-hand end of the genome. These results confirm the in vivo experiments by Bachenheimer and Darnell (1), and they indicate a common origin(s) on *Bam*HI fragment B for adenovirus transcription late in the infectious cycle. The in vitro experiments furthermore suggest that there is no arbitrary initiation of transcription in isolated nuclei and that the RNA polymerase II, which probably is utilized for viral transcription (29), elongates the transcription initiated in vivo with fidelity. To ensure that the restriction enzyme fragment hybridization patterns (Fig. 5) are not due to accumulation of unlabeled RNA sequences that would compete out the labeled nascent sequences in some gradient fractions, exhaustive hybridization to 5 μg of

TABLE 2. *Strand specificity of viral RNA synthesized in in vitro nuclei*[a]

DNA	RNA in hybrid (cpm)	Hybrid RNA (% of total)
None	276	1.20
0.5 μg of l-strand	288	1.25
0.5 μg of r-strand	6,063	26

[a] 23,000 cpm of [^{3}H]GTP-labeled RNA from isolated nuclei was hybridized for 20 h to intact unlabeled strands of Ad2 DNA as described in Materials and Methods. The hybridization mixtures were RNase treated and chromatographed on Sephadex G-100 columns, and the radioactivity in the void volume was considered to be RNA in hybrid. Less than 0.1% of mRNA labeled in vivo would hybridize under these conditions (17).

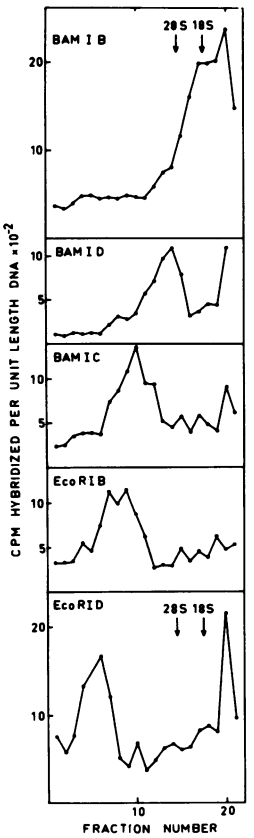

FIG. 5. *Continuous transcription of the r-strand of adenovirus DNA. Nuclei were pulse labeled for 3 min with [³H]UTP. The RNA was phenol extracted and sedimented in nondenaturing sucrose gradients. Each fraction was hybridized for 40 h to BamHI fragments B, C, D and EcoRI fragments B and D of Ad2 DNA on filters (equimolar with 1 μg of Ad2 DNA per filter). Hybridization was performed in the presence of a 200-fold excess of cold VAI and VAII RNA (27) compared with DNA on the filters. After hybridization the filters were RNase treated, and the radioactivity hybridizing to the filters represents hybridization to unit-length Ad2 DNA.*

Ad2 DNA on filters was also performed on each fraction. The results (Fig. 6A) clearly rule out the possibility that the restriction fragment hybridization would not have been carried out in DNA excess since there is only a two- to three-fold difference of hybridized RNA across the gradient. The accumulation of hybridizable low-molecular-weight RNA in Fig. 6A was mainly due to hybridization of VA RNA, since hybridization was in this case not carried out with an excess of VA RNA as in Fig. 5.

Unspecific aggregation of the in vitro transcribed RNA was furthermore controlled by cosedimenting in vivo ^{32}P-labeled cytoplasmic poly(A)+ RNA isolated late in adenovirus infection with the in vitro pulse-labeled RNA. The late viral RNA, which is 0.5 to 5 kb long (12), did not form aggregates under our centrifugation conditions and sedimented as a peak between 18S and 28S (Fig. 6B).

Elongation rate for high-molecular-weight RNA transcription. Calculation of the transcription rates in in vitro nuclei has always been arbitrary because of the difficulties in estimating the number of templates and active RNA polymerases. These drawbacks may be overcome by pulse-chase experiments, where the growth of the nascent RNA molecule is monitored by hybridization of sucrose gradient fractions to DNA probes that are much smaller than the complete transcripts, provided that processing of the RNA is negligible. Nuclei were labeled for 3 min with [³H]UTP and chased by the addition of a 50-fold excess of unlabeled UTP. Samples were taken after 3 min of labeling and after a 50-min chase period. The RNA was extracted and sedimented in sucrose gradients under nondenaturing condi-

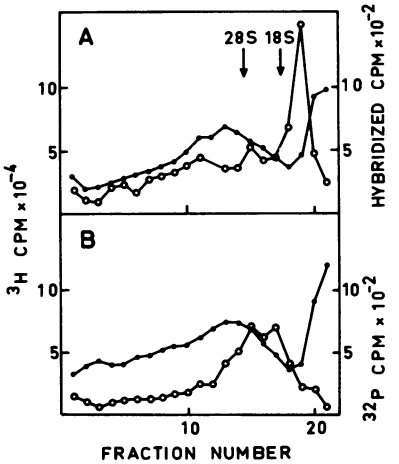

FIG. 6. *Size distribution of viral RNA sequences in pulse-labeled RNA from nuclei isolated late in adenovirus infection. Nuclei were isolated at 14 h after infection, and the RNA was labeled for 3 min in vitro with [³H]UTP. The RNA was phenol extracted and centrifuged on 15 to 30% sucrose gradients under nondenaturing conditions. (A) Total radioactivity of RNA synthesized (●). Virus-specific RNA determined by exhaustive hybridization of each fraction on filters containing 5 μg of Ad2 DNA (○). The hybridization to the filters represents hybridization to unit-length Ad2 DNA. (B) Total radioactivity of RNA synthesized (●). ^{32}P-labeled poly(A)+ viral viral mRNA (○) labeled in vivo from 12 to 16 h postinfection with inorganic ^{32}P was centrifuged in the same gradient.*

tions. The growth of the pulse-labeled RNA was monitored by hybridizing the fractions to *Bam*HI fragments (Fig. 7). The order of the *Bam*HI fragments along the linear map of the adenovirus genome is B, D, C, and A (25). The patterns of hybridization after the pulse suggest again that there is a common initiation site(s) in *Bam*HI fragment B. The differences in chain length between RNA hybridizing to a specific fragment before and after the chase may be a direct measure of the RNA chain growth during the chase, provided that no processing occurs during the chase as suggested from the hybridization patterns to the fragments emanating from the right-hand end of the genome (Fig. 7). The calculated elongation rate varied, depending on the DNA probe used (Table 3). Fragments distal (C and A) to the origin of transcription gave small or no differences in the hybridization patterns, whereas hybridization to the B and D fragments gave distinct differences in sedimentation coefficients of the RNA from pulse and the pulse-chased samples. The increase during the 50-min chase in chain length for fragment B-specific RNA was 8 kb and that for fragment D was 5 kb, which corresponds to elongation rates of 2.7 and 1.7 n/s, respectively. Considering that the initial rate of transcription is at least twice as high as the overall rate during the 50-min chase (Fig. 1), the average initial elongation rate for RNA polymerase II is calculated to be 3 to 6 n/s at 30°C. No difference in hybridization patterns for fragments C and A was found probably due to the difficulties in observing the growth of molecules that are already large at the initiation of the chase. It should be emphasized that these estimates of the elongation rates are minimal, since some undetected processing may occur. During the chase period there was a 20% loss in radioactivity from the total pulse-labeled RNA (Table 4), and sucrose gradient analysis showed that when only the high-molecular-weight RNA was taken into account, 42% of the radioactivity was acid soluble after the chase. Hybridization of total RNA to Ad2 DNA revealed that 35% of the radioactivity of viral RNA was lost during the chase. This can also be observed from the hybridization patterns to the individual fragments in Fig. 7, but in spite of the turnover the fragment-specific RNA sedimented as distinct peaks after the chase. It therefore appears that 35 to 42% of the newly synthesized high-molecular-weight RNA is degraded to non-hybridizable or acid-soluble products during the chase. The RNA that may have been released from the nuclei to the medium was always included in the analysis as described in Materials and Methods.

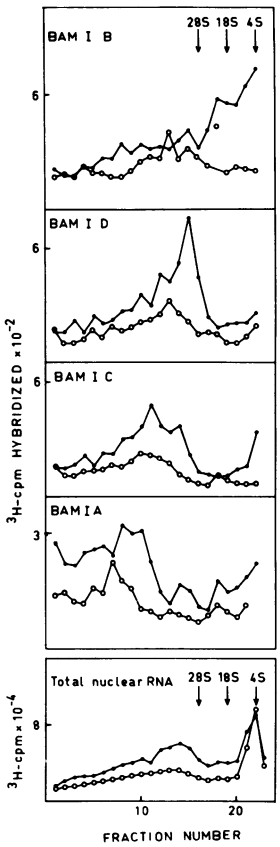

FIG. 7. *RNA chain elongation during a chase. Isolated nuclei collected at 14 h postinfection were labeled with [^{3}H]UTP for 3 min; then a sample was withdrawn, and unlabeled UTP was added to the rest of the culture in a 50-fold excess for 50 min. RNA from both samples was phenol extracted and analyzed on nondenaturing sucrose gradients, and each fraction was hybridized to different restriction fragments as described in the legend to Fig. 5. Symbols: (●) 3-min pulse RNA; (○) 50-min chase RNA.*

When the RNA from the pulse-labeled and -chased samples was analyzed on sucrose gradients under denaturing conditions (Fig. 8), most of the RNA was smaller than 8 kb. The average size of the denatured RNA was only around 2 kb, and there appeared to be only minor differences in size between the RNA obtained after the pulse and the following chase. No distinct mRNA pattern was revealed after the chase, again suggesting that processing did not occur.

DISCUSSION

In vitro nuclei isolated late in adenovirus infection of HeLa cells appear to transcribe viral RNA with fidelity compared with in vivo RNA synthesis. RNA is almost exclusively

TABLE 3. *Chain elongation of viral RNA during a chase period in isolated nuclei*

*Bam*HI restriction enzyme fragment	Label in RNA	Avg sedimentation rate of RNA (*S* value)	Avg mol wt (× 10^{-6})[a]	Length (kb)	Elongation rate[b] (n/s)	Calculated initial elongation rate[c] (n/s)
B	Pulse	12	0.37	1.1	2.7	5.4
	Chase	39	3.2	9.1		
D	Pulse	35	2.6	7.4	1.7	3.4
	Chase	46	4.3	12.3		
C	Pulse	50	5.0	14	ND[d]	ND
	Chase	54	5.8	16		
A	Pulse	67	8.5	24	ND	ND
	Chase	67	8.5	24		

[a] The molecular weights were calculated relative to marker 28S and 18S RNA in parallel gradients according to Studier (26).
[b] Mean elongation rate during 50 min of incubation.
[c] Calculated to be at least twice as high as the overall rate during 50 min of incubation (Fig. 1).
[d] ND, No elongation detectable.

TABLE 4. *Turnover of pulse-labeled RNA during a 50-min chase in in vitro nuclei*[a]

RNA prepn	Total pulse-labeled RNA (cpm × 10^{-4})	Pulse-labeled and -chased RNA (cpm × 10^{-4})	Turnover (%)
Total RNA I	197	162	18
Total RNA II	7.1	5.6	21
RNA I > 10S[b]	70	40	42
Viral RNA from II[c]	0.34	0.22	35

[a] Labeling of isolated nuclei and preparation of RNA are described in the legend to Fig. 7.
[b] Turnover of high-molecular-weight RNA from preparation I calculated from the radioactivity in the sucrose gradients.
[c] Total RNA from preparation II was hybridized exhaustively to duplicate filters containing 3 μg of Ad2 DNA. Only around 5% of the RNA radioactivity hybridizes to adenovirus DNA after short pulses.

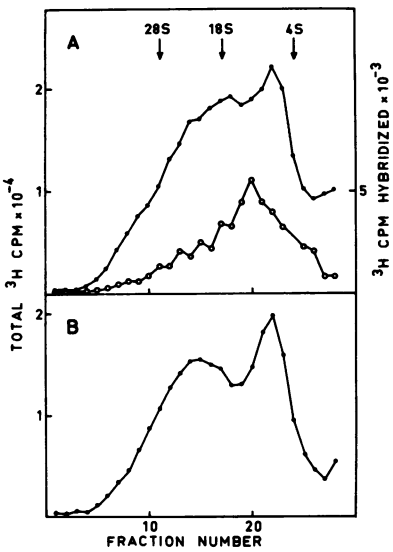

FIG. 8. *Analysis of pulse-labeled and pulse-chased RNA on denaturing sucrose gradients. Nuclei were labeled as described in the legend to Fig. 7. The RNA was denatured in 90% formamide for 2 min at 50°C, diluted to 70%, and centrifuged at 40,000 rpm for 35 h at 25°C in the Spinco SW40 rotor. (A) Total RNA radioactivity from pulse-labeled nuclei (3 min) (●). Hybridized radioactivity (○) to filters containing 5 μg of Ad2 DNA. (B) Total RNA radioactivity from pulse-labeled and -chased nuclei (3 + 50 min) (●).*

transcribed from the r-strand of adenovirus DNA. A 40-fold or higher preference for the r-strand could be demonstrated (Table 3). It has earlier been shown that newly synthesized mRNA is preferentially (100-fold) derived from the r-strand at late times in the infectious cycle (17). More recently it has been demonstrated that extracted transcription complexes from Ad2-infected cells (33) only transcribe the r-strand of the genome (C. Kedinger, personal communication). It therefore appears established that only the r-strand is transcribed at late times in the infectious cycle. The RNA synthesized in isolated nuclei is polyadenylated to around 15% (Table 1), which is lower than the 38% polyadenylation of nuclear RNA observed in vivo (18). Around 50 to 60% of the RNA synthesized in isolated nuclei appears to be virus coded, a figure that compares reasonably well with the in vivo conditions, where 40% of nuclear RNA appeared to be of viral origin (18).

It has recently been claimed that the transcription of viral RNA late in the infectious cycle gives rise to large transcripts with a common origin in the left half of the genome (1), based on a Dintzis-type experiment (4) involv-

ing short pulses followed by size fractionation of the RNA and analyses of the RNA with restriction enzyme fragment hybridization. A similar experiment with RNA from isolated nuclei reveals a common origin(s) in the left 30% of the genome and an extension of the nascent chains throughout the genome (Fig. 5 and 7). The fact that these large nascent RNA molecules are intact on nondenaturing gradients (Fig. 5 and 7) but much smaller on denaturing gradients (Fig. 8) may suggest that nicks occur during transcription within areas that are preserved in nondenaturing gradients by secondary structure. No evidence was obtained for aggregation during the size fractionation (Fig. 6). The twofold accumulation of hybridizable RNA to *Bam*HI fragment B (Fig. 5 and 7) may suggest that there are two origins within this fragment, a hypothesis that may be resolved if additional fragments in this area are analyzed. On the other hand, the size of the nascent RNA hybridizing to *Bam*HI fragment B is sufficiently large to indicate that transcription initiates around 1.5 kb from the left-hand end of the genome. An alternative explanation to the accumulation of RNA in the left-hand end may therefore involve a termination control of transcription where only a fraction of the nascent chains escapes a termination close to the end of *Bam*HI fragment B. Additional experiments are required to distinguish between these alternatives. The relative amounts of RNA hybridizing to the other fragments appear to be almost unity, suggesting that the nascent chains grow at equal rates through *Bam*HI fragments D, C, and A, which would be expected if the entire strand is transcribed as a single unit. The hybridization of RNA in the pulse-labeled experiments was obviously made in DNA excess (Fig. 6), and accumulation of unlabeled RNA in some regions of the sucrose gradients cannot have caused erratic hybridization (Fig. 6). The pulse-chase experiment (Fig. 7) reveals features of adenovirus transcription in isolated nuclei late in the infectious cycle, which may or may not have any counterpart in vivo. First, processing down to mRNA size appears not to occur, and second, around 35 to 45% of the RNA seems to turn over into a non-hybridizable form within a 50-min chase period. The fact that viral RNA accumulates in the nuclei at late times in vivo with a 3.5- to 4-fold abundance in nuclei over cytoplasm (5, 18) may suggest that processing is extremely slow once the polyribosomes have accumulated large amounts of viral mRNA. The significant turnover may suggest that nuclear RNA is rapidly degraded by a combined action of endo- and exonucleases in vitro. Similar experiments cannot be performed in vivo because of the large pool sizes of nucleotides in mammalian cells, which are prohibitive for pulse-chase experiments. It is tempting to speculate that if the turnover is selective in vivo, leaving some specific regions of the RNA intact, it may explain the drastic difference in abundance of RNA from different regions of the genome observed in adenovirus-infected cells (5).

In conclusion, the isolated nuclei obviously provide an additional and powerful tool to study the details of adenovirus transcription, and we are at present attempting to identify the initiation sites for viral RNA synthesized by polymerases II and III.

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