Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 160–164, January 1979
Biochemistry

RNA synthesis in isolated nuclei: *In vitro* initiation of adenovirus 2 major late mRNA precursor

(transcription/mRNA "cap"/RNA polymerase II/RNA processing)

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Communicated by Howard Green, October 12, 1978

ABSTRACT **We have analyzed the RNA produced *in vitro* by incubating nuclei from HeLa cells infected with adenovirus serotype 2. Our results show that adenovirus-specific RNA is produced at a linear rate for up to 2.5 hr. Hybridization analysis of RNA produced in nuclei isolated 18 hr after infection indicated that transcription begins at the "late promoter" at map position 16.5. Sequence analysis of the 5′ termini of the *in vitro* transcripts showed that this system initiates RNA chains *de novo* at the correct promoter and that the 5′ terminus is capped.**

The process of regulation of gene expression at the level of RNA transcription is reasonably well understood in prokaryotic systems. The enzymes involved in mRNA synthesis and the factors that regulate these enzymes have been well studied (1). These findings have in large part been due to the availability of *in vitro* systems capable of synthesizing mRNA. In contrast, little is known about the biochemistry of these processes in eukaryotic systems. It is well established that in eukaryotes there are distinct RNA polymerases that catalyze the synthesis of ribosomal RNA (RNA polymerase I), mRNA (RNA polymerase II), and tRNA and 5S RNA (RNA polymerase III) (2). To date, however, there have been no reports demonstrating the synthesis of mRNA, or its precursor, *in vitro*.

In an attempt to gain insight into the biochemical mechanisms involved in mRNA biosynthesis, systems containing isolated nuclei from mammalian cells have been used (3, 4). It has been reported that initiation of RNA chains dependent upon RNA polymerase II can take place in isolated nuclei (5). Other recent results have suggested that large nuclear transcripts are generated *de novo* in isolated nuclei (6).

In order to clarify the nature of the transcription observed in isolated nuclei, we turned our attention to the activity of nuclei isolated from cells during the late phase of infection by adenovirus serotype 2 (Ad2). This system has many advantages over systems from uninfected cells. Both RNA polymerase II and RNA polymerase III catalyze the synthesis of viral specific RNA in these nuclei, and the *in vivo* strand specificity of transcription is retained (7–10). The product of RNA polymerase III activity on viral DNA is two small defined RNAs (VA RNAs) and this polymerase will initiate transcription of these small RNAs upon incubation of nuclei extracted from late infected cells (11). The RNA polymerase II transcription units in these cells have also been defined. Specifically, the origin of the transcription unit leading to the synthesis of the majority of late mRNAs has been localized by various methods (12–15), all of which place the site for initiation of transcription at approximately 16.5% on the Ad2 genome. In keeping with these results is the finding of a unique 5′-end sequence on most, if not all, late viral cytoplasmic mRNAs (16). This undecanucleotide contains a 7-methylguanosine "cap" and has been identified on the 5′ end of two purified mRNAs as well as of nuclear pre-mRNA (17, 18). The DNA sequence encoding this undecanucleotide has recently been localized in the adenovirus genome, thereby confirming the assignment of the 16.5% location as a "promoter" site for late transcription (18).

In this report, we demonstrate that nuclei isolated from Ad2-infected cells 18 hr after infection are capable of supporting initiation of transcription. The *in vitro* transcript begins at the promoter for the major late transcription unit, and it contains the capped 5′-undecanucleotide.

MATERIALS AND METHODS

Cells, Virus, and DNA. HeLa cells were propagated in suspension culture in Eagle's minimal essential medium supplemented with 5% horse serum. Cells at a concentration of 4–6 $\times 10^5$ cells per ml were infected with Ad2 (25 plaque-forming units per cell). Infections were carried out by adsorbing the virus to the cells after concentration to 4–6 $\times 10^6$ cells per ml. After a 1-hr incubation at 37°C, the cells were diluted to 4×10^5/ml with fresh medium. For preparation of nuclei, cells were harvested 18 hr after infection. DNA was extracted from purified virions as described (19). DNA was digested with *Bam*HI, and the DNA fragments were separated and recovered as described (20).

Preparation of Nuclei and *In Vitro* Incubation. Nuclei were prepared from infected cells essentially by the method of Mory and Gefter (3). Nuclei were incubated *in vitro* for RNA synthesis as described (3, 6), except that the concentrations of the labeled ribonucleoside triphosphate were adjusted as indicated in the figure legends. No cytoplasmic extract was added. Preliminary experiments showed that addition of such extracts did not qualitatively affect the RNA synthesized in any way we could detect.

Purification of RNA. Nuclei were resuspended (250 μl/10^7 nuclei) in 0.6 M NaCl/10 mM Tris, pH 7.5/20 mM $MgCl_2$ containing 60 μg of DNase I [Worthington, RNase free; the DNase was treated with iodoacetate (21) to inactivate any residual RNase] per ml and incubated for 10 min at 25°C with shaking (22). Urea was then added to 4 M, sodium dodecyl sulfate ($NaDodSO_4$) to 1.0%, and EDTA to 20 mM. An equal volume of phenol/chloroform/isoamyl alcohol (1:1:0.05, vol/vol) was added. After mixing and centrifugation the aqueous phase was decanted, and the organic phase was reextracted with 7 M urea/0.35 M NaCl/10 mM Tris, pH 8.0/2 mM EDTA/0.5% $NaDodSO_4$ (23). The aqueous phases were pooled, reextracted, and precipitated with ethanol. The RNA was resuspended in 10 mM Tris, pH 8.0/1 mM EDTA/0.5% $NaDodSO_4$ and passed through a 15 $\times$ 0.6 cm column of Sephadex G-100. RNA in the excluded volume was precipitated with ethanol and the pellet was washed with 95% ethanol. RNA to be used for

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Abbreviations: Ad2, adenovirus serotype 2; $NaDodSO_4$, sodium dodecyl sulfate; kb, kilobases.

hybridization was resuspended in 0.2% Sarkosyl/1 mM EDTA, pH 8.0, and stored at −20°C.

RNA·DNA Hybridization. Hybridizations were carried out at approximately 5-fold DNA excess in 80% formamide/0.04 M 1,4-piperazinediethanesulfonic acid, pH 6.5/0.4 M NaCl/1 mM EDTA as described (24, 25). Treatment of the hybridized nucleic acids with nuclease S1 (Boehringer) or RNase A and agarose gel electrophoresis were performed as described in the figure legends and ref. 24.

Nuclease Digestions. Nuclear RNA to be digested with RNase T1 was resuspended in 10 mM Tris, pH 7.5/1 mM EDTA at a concentration of 5 mg/ml, heated to 100°C for 3 min, and then quenched in ice water. RNase T1 (Calbiochem) was added to obtain an enzyme/substrate ratio of 1:5, and reaction mixtures were incubated for 2 hr at 37°C. Digestions of RNA eluted from homochromatography plates with RNase T2 or U2 (Calbiochem) were carried out as described by Barrell (26). Digestions of RNA eluted from DEAE paper with snake venom phosphodiesterase (Sigma) or nuclease P1 (Calbiochem) were performed as described (17), except that in both cases the reaction products were analyzed by reelectrophoresis on DEAE paper at pH 3.5. Radioactive nucleotides were visualized by exposure to Kodak XR-5 film at −70°C, using two Du Pont Cronex "Lightning Plus" intensifying screens.

Analysis of Oligonucleotides by Dihydroxyboryl Cellulose Chromatography. RNA digested with RNase T1 was phenol extracted, ether extracted, and adjusted to 0.6 M KCl, 0.05 M *N*-methylmorpholine (pH 8.5), and 20% ethanol. The RNA was then bound to a column of dihydroxyboryl cellulose and eluted, concentrated, and desalted as described (16). The oligonucleotides were fractionated by two-dimensional fingerprint analysis, using homomixture C in the second dimension (26).

RESULTS

Properties of RNA Synthesized *In Vitro*. It has been shown (3) that nuclei prepared from murine plasmacytoma cells are capable of supporting RNA synthesis *in vitro* at a linear rate for up to 3 hr of incubation. Using essentially the same procedure, we prepared nuclei from Ad2-infected HeLa cells 18 hr after infection. Synthesis of RNA proceeded at an approximately linear rate for up to 150 min of incubation (Fig. 1). The rate of accumulation of RNA was about 10–20 pmol of UMP incorporated per min per 10^7 nuclei. This rate is such that after a 2-hr *in vitro* incubation an amount equivalent to approximately 10–20% of the total endogenous Ad2-specific RNA had been synthesized *in vitro*. A fraction of the total RNA synthesized does not sediment with the nuclei. All of the characterization of RNA in this report deals only with the intranuclear RNA.

RNA synthesized *in vitro*, sampled after either 15 or 120 min of synthesis, had a broad sedimentation distribution under denaturation conditions. Molecules up to 25 kilobases (kb) long were observed (not shown).

It has been shown (7–10) that nuclei prepared from Ad2-infected cells synthesize RNA complementary to the viral genome. In agreement with these reports, 52% of the RNA synthesized in nuclei prepared and incubated under conditions described here was virus-specific (Table 1). Furthermore, greater than 90% of the virus-specific RNA appeared to be the result of RNA polymerase II catalysis, as judged by sensitivity to α-amanitin at 0.5 μg/ml.

Analysis of the 5′ End of RNA Transcripts. In order to further characterize the nature of *in vitro* transcription, we analyzed the 5′ ends of the RNA synthesized *in vitro*. We used a modification of a technique (24) in which RNA is hybridized to purified restriction fragments of Ad2 RNA under conditions that favor DNA·RNA hybrid formation and treated with nuclease S1, and then the resistant hybrids are analyzed by agarose gel electrophoresis.

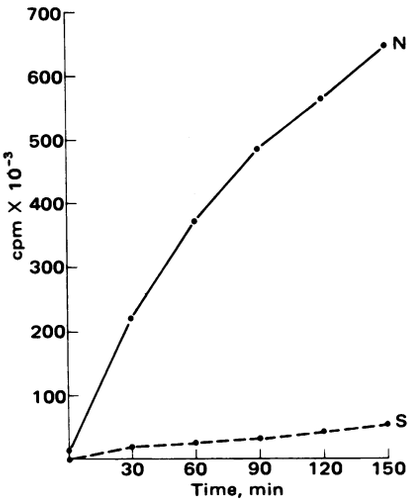

FIG. 1. Kinetics of *in vitro* RNA synthesis. Nuclei (8×10^6/100 μl reaction mixture) were incubated in standard reaction mixtures containing 2.5 μCi of [α-^{32}P]GTP (1 Ci/mmol). Reactions were terminated at the indicated times by quenching on ice, and nuclei (N) were separated from supernatants (S) by centrifugation. Nuclei were resuspended in 7 M urea/0.5% $NaDodSO_4$/10 mM EDTA/50 mM NaOAc, pH 5.2, both fractions were precipitated with trichloroacetic acid, and radioactivity was determined by liquid scintillation counting.

Radioactive RNA was hybridized to *Bam*HI fragment B (0–29.1 map units), treated with nuclease S1, and analyzed by gel electrophoresis (Fig. 2). The length of the major protected fragment (12.5% of Ad2 length) is consistent with the 5′ end point of the transcripts being at position 16.5 (29.1–12.5). The assignment of the exact location was made by using overlapping DNA restriction fragments. In addition to the DNA·RNA hybrid of approximately 12.5%, we reproducibly observed a hybrid of 9.5% of the genome length, the 5′ end point of which is located at map position 19.5.

The amount of RNA recovered in a hybrid with *Bam*HI fragment B was approximately equimolar with the amount hybridizing to fragments extending toward 100%, as judged by

Table 1. α-Amanitin sensitivity of *in vitro* synthesized RNA

α-Amanitin, μg/ml	RNA synthesized, %	
	Total	Ad2-specific
0	100	52
0.5	23	3.5
200	3	<0.2

The amount of RNA synthesized *in vitro* (4×10^6 nuclei in 50-μl reaction mixtures, 2 μCi of [α-^{32}P]CTP, 1 Ci/mmol, as the radioactive precursor) in the presence of the indicated concentration of α-amanitin was determined by trichloroacetic acid precipitation. Total RNA synthesis was measured by trichloroacetic acid precipitation of entire reaction mixtures after 1 hr of synthesis. Ad2-specific RNA synthesis was measured after purification of RNA from reaction mixtures and solution hybridization to a 10-fold excess of Ad2 DNA. The amount of RNA synthesized complementary to Ad2 DNA was found by measuring the amount of RNA remaining precipitable after incubation of the hybridized nucleic acids with pancreatic RNase (10 μg/ml) in 10 mM Tris, pH 7.5/300 mM NaCl/2 mM EDTA at 37°C for 30 min. The radioactivity that remained precipitable when Ad2 DNA had been replaced by λ DNA in the hybridization reactions was subtracted. Values in the table are expressed as percentages of the total amount of RNA synthesized in the absence of α-amanitin (approximately 4×10^5 dpm).

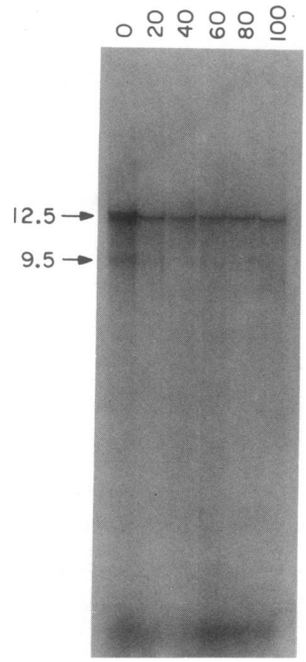

FIG. 2. Synthesis *in vitro* of the 5′ end of the Ad2 major late transcription unit for an extended period of time. Nuclei (6×10^6) were incubated in 80-μl reaction mixtures. At the times (minutes) indicated above each track, 15 μCi of [α-^{32}P]UTP (120 Ci/mmol) was added to each reaction; 20 min later, nuclei were separated by centrifugation and RNA was isolated from them. An aliquot of RNA was hybridized to 0.5 μg of the *Bam*HI fragment B of Ad2 DNA (0–29.1 map units) and digested with nuclease S1, and the DNA·RNA hybrids were analyzed by electrophoresis through 1.2% agarose slab gels (24). The numbers at the side indicate the size of the DNA·RNA hybrids expressed as percentages of the length of intact Ad2 DNA (10.0 is approximately 3500 base pairs).

comparison of the intensity of DNA·RNA hybrids obtained following electrophoresis of the S1-resistant molecules (unpublished data). Thus, it appears that a large fraction of all transcription seen *in vitro* either extends molecules initiated at position 16.5 or initiates synthesis at position 16.5.

We used this hybridization technique to obtain evidence for *in vitro* initiation of transcription at position 16.5. Molecules just initiated *in vivo* and then elongated *in vitro* to a length greater than approximately 4 kb will give rise to the DNA·RNA hybrid band shown in Fig. 2. The chain elongation rate measured in this system was approximately 400–500 nucleotides per minute, as determined by measuring the rate of accumulation of "fully dense" RNA when Hg UTP is used as a precursor (6). Thus, labeling for 20 min should allow all molecules previously initiated to grow to lengths greater than 4 kb. If we allow elongation to proceed for 20 min in the presence of nonradioactive precursors and then add α-^{32}P-labeled UTP for 20 min, radioactive molecules hybridizing to the segment 16.5–29.1 should be ones that are either elongating slowly or have initiated *in vitro*. This analysis was extended to labeled RNAs made during 20-min intervals for up to 120 min. Fig. 2 shows that radioactive molecules with 5′ ends at position 16.5 were synthesized throughout the 120-min incubation period. These results strongly suggest that the nuclei in this system are supporting *in vitro* chain initiation. However, to rule out the possibility that there is a large pool of "preinitiated" chains that are simply being elongated *in vitro*, we analyzed the radioactivity incorporated into the first 11 nucleotides of the transcripts.

Analysis of the 5′ Undecanucleotide of *In Vitro* Transcripts. Incorporation of radioactive phosphorus from the β or γ position of ATP or GTP into polynucleotide is an accepted way of demonstrating *de novo* initiation (11). However, a triphosphorylated-terminated RNA synthesized by RNA polymerase II has not yet been documented, nor has a known mRNA-like transcript been isolated containing a triphosphorylated terminus. Therefore, we attempted to demonstrate the incorporation of radioactivity *in vitro* into the capped undecanucleotide found on the 5′ end of Ad2 late mRNAs (16, 17). This oligonucleotide is encoded in the DNA sequence at position 16.5 (18). To this end, total nuclear RNA (labeled *in vitro*) was hydrolyzed with RNase T1, fractionated on a column of dihydroxyboryl cellulose, and analyzed by fingerprinting.

A fingerprint of total RNA synthesized in the *in vitro* system using [α-^{32}P]GTP is shown in Fig. 3*A*. A much simpler fingerprint was given by the oligonucleotides eluted from dihy-

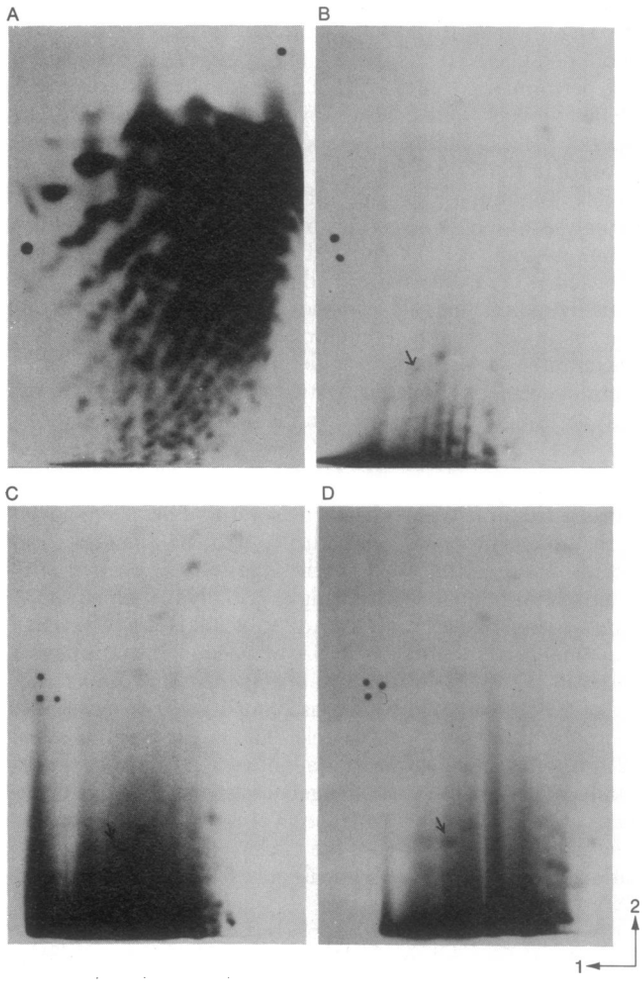

FIG. 3. Dihydroxyboryl cellulose-selected T1 oligonucleotides synthesized *in vitro*. Reaction mixtures contained 2×10^7 nuclei in 200 μl. The T1 oligonucleotides displayed in *A* and *B* were obtained from a reaction mixture containing 1 mCi of [α-^{32}P]GTP (200 Ci/mmol), those in *C* were from one containing 600 μCi of [α-^{32}P]UTP (120 Ci/mmol), and those in *D* were from one containing 600 μCi of [α-^{32}P]CTP (120 Ci/mmol). After 2-hr incubation *in vitro*, nuclei were pelleted and RNA was extracted. RNA was digested with RNase T1 and selected on columns of dihydroxyboryl cellulose. Approximately 0.8% of the radioactivity applied to the columns was bound and eluted. The oligonucleotides were then purified and displayed by fingerprint analysis as described (16, 26). *A* shows the fingerprint of an aliquot (0.5%) of the RNA prior to selection on dihydroxyboryl cellulose. All spots in *B*, *C*, and *D* that migrated slower than the yellow dye (spot of radioactive ink) in the second dimension were eluted and digested with RNase T2. The only spot in each fingerprint with detectable levels of T2-resistant radioactivity is indicated by an arrow.

droxyboryl cellulose (Fig. 3*B*). All spots migrating slower than the yellow dye in the second dimension were eluted and digested with RNase T2. Only the spot indicated by the arrow (Fig. 3B) reproducibly contained an oligonucleotide partially resistant to T2 (Fig. 4, track 1). This oligonucleotide yielded m^7GMP when it was digested with snake venom phosphodiesterase (not shown). An additional spot, that also yielded a low level of a T2-resistant radioactivity and migrated slightly faster in the first dimension than the spot marked by the arrow, has occasionally been detected (Fig. 3 *C* and *D*; the spot immediately to the left of the one indicated by the arrow). This observation is consistent with previous findings (16) and has not been investigated further. From the known composition of the undecanucleotide (16), we expected that both the T1-resistant oligonucleotide and the T2-resistant structure generated from it could also be labeled with CTP or UTP. In fact, the spots marked with arrows in Fig. 3*C* and *C* also contained the T2-resistant oligonucleotide (Fig. 4, tracks 3 and 4). In addition to the resistant oligonucleotides shown in Fig. 4, larger than expected amounts of radioactivity can be detected as mononucleotides. In order to quantitate the relative amounts of radioactivity in the cap and mononucleotides, the T1 spots therefore had to be repurified prior to RNase T2 digestion.

To this end, we isolated the oligonucleotide labeled with CTP from a two-dimensional fingerprint and incubated it with RNase U2. Because the only A residue present in the undecanucleotide is 2′-*O*-methylated and therefore resistant to RNase U2, the T1-resistant oligonucleotide we isolated should be completely resistant to RNase U2. Fig. 4 (track 5) demonstrates the presence of a U2-resistant undecanucleotide.

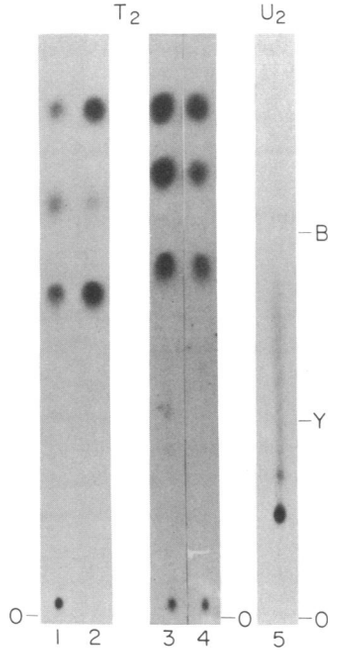

FIG. 4. Sensitivity of *in vitro* synthesized Ad2 5′-terminal T1 oligonucleotide to nucleases. Tracks 1–4 show RNase T2 digests analyzed by electrophoresis at pH 3.5 on DEAE paper. Track 5 shows a RNase U2 digestion, analyzed by homochromatography on a DEAE-cellulose thin layer. The oligonucleotides analyzed were as follows: track 1, spot indicated by arrow in Fig. 3*B*; track 2, typical spot from the fingerprint shown in Fig. 3*B*; track 3, spot indicated by arrow in Fig. 3*C*; track 4, spot indicated by arrow in Fig. 3*D*; and track 5, T1 oligonucleotide of Table 2. O, origin in each case; B and Y, positions to which the blue and yellow dyes migrated during homochromatography. The radioactive spots with greatest mobility in tracks 1–4 are pU, preceded by pC and then pA and pG, which are not separated by the electrophoretic conditions used. The T2-resistant structure barely migrated from the origin.

Table 2. Nearest-neighbor analysis of "capped" T1 undecanucleotide synthesized *in vitro*†

Nucleotide analyzed	^{32}P, dpm	Yield	
		Experimental	Theoretical
Up	202	2.9	3.0
Cp	73	1.1	1.0
Gp	69	1.0	1.0
($m^7Gppp^*A_mpCp$)‡	77	1.1	1.0

The Ad2 5′-terminal T1 oligonucleotide synthesized *in vitro* (3×10^7 nuclei in a 300-μl reaction mixture) with [α-^{32}P]CTP (1.0 mCi, 120 Ci/mmol) as the radioactive precursor was purified, treated with RNase U2, repurified, digested with RNase T2, and analyzed. Regions of the DEAE paper corresponding to the indicated nucleotides were quantitated by liquid scintillation counting.

† Nuclease P1 digestion of a small aliquot of the α-CTP labeled RNA gave rise to greater than 95% of the radioactivity in pC (results not shown). This finding proves that exchange of label into other nucleotides did not occur to a detectable level, thereby validating the nearest-neighbor analysis presented here.

‡ We have not determined that the second nucleotide of this trinucleotide is A_m but rather deduced it from our results combined with previously published experiments (16).

Knowing the sequence of the undecanucleotide, $m^7Gppp^*A_mC$-U-C-U-C-U-U-C-C-G-(C) (ref. 18; R. Lockard, personal communication), the nearest-neighbor results can be predicted. The repurified material was therefore digested with RNase T2 and the products were resolved on DEAE paper. Table 2 presents the nearest-neighbor analysis obtained, which is in excellent agreement with the predicted values. In addition, digestion of the CTP-labeled T2-resistant moiety with nuclease P1 yielded exclusively pC, proving that the second nucleotide from the 5′-terminal m^7G is C (data not shown). Thus we conclude that radioactivity is incorporated into the second nucleotide and then the next nine nucleotides of the *in vitro* transcripts and that this corresponds exactly to the 5′ end detected *in vivo*.

By quantitating the amount of radioactivity derived from CTP incorporated into the undecanucleotide relative to the amount incorporated into the total RNA transcript, we can determine the number of chains initiated: 52% of the *in vitro* transcription is Ad2 specific (Table 1), all (>90%) of this transcription is from the 25-kb major late transcription unit (14, 15), and the RNA transcript is 28% C (27). From these numbers we calculate that the theoretical yield of the capped undecanucleotide should be 0.045% of the total radioactivity incorporated, if all the transcription observed is into chains intiated *in vitro*. After correcting for losses [the major one being an estimated 30% recovery on the dihydroxyboryl cellulose column (17)], we found, in the experiment described in Table 2, that 0.0052% (6.3×10^2 cpm/1.2×10^8 cpm) of the radioactivity incorporated into RNA was in the purified undecanucleotide. Therefore we estimate that at least 12% of the observed transcription is onto chains initiated at the correct promoter *in vitro*. Because our assay detects only transcripts that have been capped and methylated correctly, as well as initiated, this number may be an underestimate.

DISCUSSION

We have presented evidence that nuclei prepared from cells infected with Ad2 are capable of supporting RNA chain initiation *in vitro* at the major late promoter. Furthermore, this RNA is capped and methylated correctly. Only a cap 1 structure (m^7Gppp^*AmpCp) is produced by the isolated nuclei (Fig. 4), although Ad2 late mRNAs have been shown to contain a mixture of cap 1 and cap 2 ($m^7Gppp^*AmpCmpUp$) structures (16). A likely explanation for this discrepancy is that conversion of

cap 1 to cap 2 is a cytoplasmic reaction and therefore does not occur in isolated nuclei. This has in fact been shown to be the case in the synthesis of L cell mRNA cap structures (28).

In addition to the RNA species originating at map position 16.5, we have observed molecules produced *in vitro* that contain 5′ termini at position 19.5 (Fig. 2). This 5′ end may result from post-transcriptional "splicing" of the transcript begun at position 16.5, because position 19.5 is the position of the first splice point found in late mRNA (12, 13). We have also occasionally observed 5′ termini at position 26.5, which corresponds to the final splice point for creation of the tripartite leader sequence. These observations are in keeping with the conclusion that the additional termini are a result of *in vitro* post-transcriptional processing (splicing) of the nuclear transcripts.

Comparison of the RNase T1 fingerprints of RNA labeled with [α-^{32}P]GTP, -UTP or -CTP (Fig. 3) shows that the amount of label in the capped undecanucleotide is greatest with CTP as the radioactive precursor and least with GTP. However, the amount of radioactivity found in RNase T2-resistant material is equivalent in each case (Fig. 4). These results are consistent with the known base composition and structure of the capped undecanucleotide (16, 18) and show that the *in vitro* synthesized oligonucleotide contains equimolar amounts of all its constituent nucleotides. In particular, the amount of radioactivity in m^{7}GMP is equivalent to the amount of radioactivity in any other single nucleotide. Although our results show that a substantial fraction of the RNA chains analyzed must be initiated *in vitro*, a large amount of the transcription observed arises from elongation of nascent chains initiated *in vivo*. Because an enzymatic activity capable of capping such nascent RNA chains has been described (29), the possibility existed that, when [α-^{32}P]GTP was used as precursor, label could be incorporated into the undecanucleotide by capping of RNA chains that had been initiated *in vivo*. However, such a situation should result in a greater-than-equimolar amount of label in m^{7}GMP. Therefore, our results indicate that such "post-transcriptional" capping does not occur at detectable levels. One interpretation of this result is that capping and transcription initiation are tightly linked. Consistent with this model are results that indicate that the capping reaction that occurs during the synthesis of cytoplasmic polyhedrosis virus mRNA may be "pretranscriptional"—i.e., occurring before formation of the first phosphodiester bond (30). Furthermore, analysis of the 5′ termini of nascent RNA chains in L cells indicates that such molecules are capped extremely early during the course of their synthesis (31).

Our results show that the isolated nuclei system we describe is capable of supporting correct initiation of the major Ad2 late transcription unit *in vitro*. In addition, recent results (unpublished data) show that virtually the entire transcription unit is synthesized at high levels for extended periods of time. Furthermore, we have obtained evidence that discrete 3′ end points, which correspond to the known 3′ end points of mRNA families found *in vivo* (32), are generated in the *in vitro* system. Thus, we think that the *in vitro* system described here will be useful in studying all the reactions that contribute to the production of mature mRNA molecules.

We thank E. Ziff, R. C. C. Huang, and R. Lockard for communicating their results prior to publication and S. Huang for excellent technical assistance. This research was supported by American Cancer Society Grant NP-6G and Grant AIi3357-03 from the National Institutes of Health.

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