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Initiation of transcription in nuclei isolated from adenovirus infected cells

Björn Vennström, Ulf Pettersson and Lennart Philipson

Department of Microbiology, Biomedical Center, Uppsala University, Uppsala, Sweden

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ABSTRACT

Initiation of adenovirus transcription was analyzed by incubation of isolated nuclei from virus infected cells in the presence of β-^{32}P GTP or β-^{32}P ATP. Nucleotide analysis of RNA from nuclei incubated with β-^{32}P GTP shows that the label is incorporated exclusively into pppGp and ppGp. Under similar incubation conditions, the label from β-^{32}P ATP was incorporated primarily into the 5' phosphate of 5', 3' mononucleoside diphosphates, but label was also detected in pppAp, pppGp and in the 3' nucleoside monophosphates. Analysis of RNA, synthesized in the presence of different concentrations of α-amanitin, shows that only RNA polymerase III initiates virus specific transcription in isolated nuclei. The virus specific transcripts containing pppAp and pppGp in their 5' termini were identified as the 5.5S and 5.2S viral RNA species by hybridization and finger printing.

INTRODUCTION

Isolated nuclei from eukaryotic cells have been useful to study DNA replication and transcription. Reichard et al. (13) investigated the RNA primer for polyoma DNA synthesis in isolated nuclei. They were able to demonstrate that the RNA primer initiated randomly on the genome during DNA synthesis. Transcriptional studies have assigned the three mammalian RNA polymerases to their respective functions by virtue of their differential sensitivities to the drug α-amanitin (2-5). Transcription of ribosomal RNA, 5S RNA and adenovirus RNA occurs from the correct DNA strands in isolated nuclei (6-8). Initiation of transcription in isolated nuclei has only been demonstrated for the RNA polymerase III products, 5 S RNA (7) and the adenovirus coded 5.5S and 5.2S RNA species (9,10). The viral products comprise two stable species (10,11,12), of which one has been sequenced (10) and shown to have two different 5' terminal sequences (1). The origin of both species on the viral genome has been determined.

This paper describes studies on the initiation of adenovirus specific transcription by introducing label in the 5' termini of RNA with β-^{32}P

labeled purine triphosphates in isolated nuclei. Specific terminal labeling is shown only to occur with β-^{32}P GTP. Initiated viral RNA was analyzed by gel electrophoresis, finger printing and hybridization. The 5.2S RNA and the two 5.5S RNA species are the only viral transcripts which initiate in isolated nuclei and their 5' terminal sequences agree with those generated in vivo.

MATERIALS AND METHODS

Virus infection and cell fractionation

HeLa cells were infected with adenovirus type 2 as described previously (8). Nuclei were isolated from infected cells late in infection by Dounce homogenization of hypotonically swollen cells (8).

RNA transcription in isolated nuclei

^{3}H-labeled nucleoside triphosphates and $^{32}PO_4$ were purchased from Amersham Radiochemicals.

The conditions for RNA synthesis were essentially the same as previously described (8). When labeling with β-^{32}P ATP the concentration of the precursor was kept at 100 μM with a specific activity of 10-60 Ci/mM. Labeling with β-^{32}P GTP was carried out at a concentration of 50 μM at a specific activity of 50-100 Ci/mM. β-^{32}P labeled ATP and GTP were made by exchange of ^{32}Pi with the corresponding diphosphates and subsequent phosphorylation of the diphosphates with nucleoside diphosphokinase or pyruvate kinase as outlined by Reichard et al. (13). RNA was purified by extraction of the incubation mixtures with phenol as described in the accompanying paper (1).

Restriction endonucleases and cleavage of Ad2 DNA

EndoR BamHI was purified as previously described (10). EndoR Hinf and endoR BalI were generous gifts from Dr. M.B. Mathews. The digestions of ad2 DNA were carried out at 37°C in 6 mM Tris-HCl pH 7.9, 6 mM β-mercaptoethanol and 6 mM $MgCl_2$. Digestions with endoR SmaI, purified as described by Greene and Mulder (personal communication), were performed at 30°C in 0.1 M Tris-HCl pH 9.0, 6 mM β-mercaptoethanol and 6 mM $MgCl_2$. The fragments were separated on 1 % or 1.4 % agarose slab gels in 90 mM Tris-borate and 2.5 mM EDTA pH 8.3. DNA fragments in the gels were denatured and transferred to nitrocellulose sheets (14).

Nucleic acid hybridization

Preparative hybridization with labeled RNA and total ad2 DNA was carried out as described previously (8) and RNA was eluted from filters as

described in the accompanying report (1).

Hybridization of RNA to restriction enzyme fragments, immobilized on nitrocellulose strips, was carried out in 6 x SSC and 0.2 % SDS at 65°C for 1 or 2 days. The strips were washed with 2 x SSC at 65°C and treated with 20 µg/ml of pancreatic RNase. Hybridization to specific fragments was detected by autoradiography.

Acrylamide gel electrophoresis

Electrophoresis in 12 % polyacrylamide gels containing 7 M urea was carried out as described by Maniatis et al. (15).

Nucleotide analysis

Digestions with pancreatic RNase, RNase T1 and RNase T2 were carried out as described (1). Endonuclease P1 (Yamasa Shoyu Co) was used in 50 mM NH_4CO_3 pH 6.0 at an enzyme to substrate ratio of 1/5 (w/w). Digestions were carried out at 37°C for 1 hour.

Two-dimensional separation of (oligo)nucleotides were performed as described in detail by Volckaert et al. (16). High voltage electrophoresis on cellulose acetate paper was employed in the first dimension, and homochromatography on DEAE or PEI thin layer plates in the second dimension. Thin layer chromatography on PEI plates was done by stepwise development with 0.3 M, 0.5 M and 1.5 M LiCl in 7 M urea, 10 mM Tris pH 7.8, and 2 mM EDTA.

Oligonucleotides were eluted from the thin layers and the labeled 5'terminal nucleotides identified by redigestion with endonuclease P1 and stepwise chromatography on PEI thin layers using 0.5 - 1.5 M LiCl in 1 M HCOOH as solvent.

Autoradiography was carried out as described in the preceeding report (1).

RESULTS

Initiation of RNA transcription in isolated nuclei

In order to specifically study initiation of transcription it is desirable to introduce radioactive label exclusively in the 5'-terminal nucleotide by the use of γ- or β-^{32}P labeled nucleotide triphosphates as precursors. Nucleoside triphosphates are unable to penetrate intact cells and therefore in vitro systems are mandatory. Preliminary experiments with γ-^{32}P labeled ATP or GTP in isolated nuclei failed, however, to show specificity in the labeling of 5' termini of RNA. This is due to phosphate exchange which may lead to incorporation of label into internal positions of the transcript. This exchange should be prevented by the use of β-labeled

nucleoside triphosphates as precursors. Therefore, nuclei isolated 14 hours after ad2 infection were incubated in vitro in the presence of β-^{32}P labeled ATP or GTP. RNA synthesis was allowed to proceed for 45 minutes and the RNA was then extracted and TCA insoluble radioactivity was determined. Approximately 2×10^{-5} % of the β-^{32}P ATP and 5×10^{-3} % of the β-^{32}P GTP were incorporated into RNA. Hybridization of the labeled RNA to filters with immobilized ad2 DNA showed that around 10 % of the β-^{32}P ATP and 5 % of the β-^{32}P GTP labeled RNA hybridized to nitrocellulose filters with 50 µg ad2 DNA. However, these values may represent minimum figures since the viral 5.5S RNA is an abundant species among the adenovirus transcripts and consequently produced in large amounts during labeling with β-^{32}P GTP. Its specific activity may therefore be too low to be quantitatively hybridized under these conditions.

In order to further characterize the labeled adenovirus specific RNA, the RNA was eluted from the filters and digested with RNase T2. The labeled nucleotides were separated by electrophoresis and thin layer chromatography on PEI plates. As shown in Fig. 1A and Table 1 more than 95 % of the β-^{32}P GTP label was incorporated into 5'-terminal nucleotides with 85 % in pppGp and 15 % in ppGp. In some experiments traces of label (<5 %) was also recovered in guanosine 5', 3' mononucleoside diphosphate (pGp) and in the 3' nucleosidemonophosphates. The identity of the guanosinetetraphosphate was verified by chromatography in two systems with unlabeled pppGp as a marker. Digestions were also carried out with bacterial alkaline phosphatase, which generated labeled inorganic phosphate, and with nuclease P1, the 3' phosphatase activity of which generated labeled pppG and unlabeled Pi. The RNase T2 fingerprint from β-^{32}P ATP labeled adenovirus specific RNA isolated in the same way shows a different pattern (Fig. 1B). The label is mainly recovered in 5', 3' mononucleosidediphosphates (67 % of the radioactivity) with a preference for pAp. Some label was detected in 3'-nucleosidemonophosphates (11 %) and in pppAp (17 %) and pppGp (5 %) as shown in Table 1. The identity of the labeled nucleotides was established by cochromatography with unlabeled markers. Nuclease P1 digestion of pAp generated exclusively labeled adenosine 5'-monophosphate, providing proof that the label was confined to the 5' position. Due to the low amounts of radioactivity in the other labeled nucleotides it was impossible to carry out further enzymatic digestions. The different labeling pattern with β-^{32}P ATP compared to that with β-^{32}P GTP is unclear and will be studied separately.

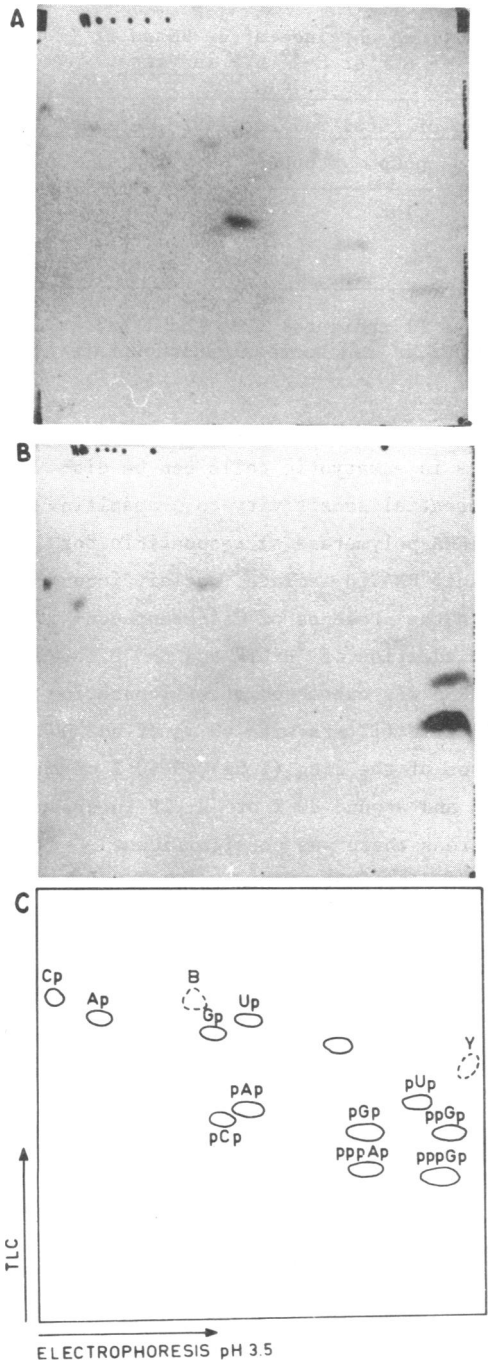

Figure 1. Nucleotide analysis of adenovirus RNA labeled with β-^{32}P ATP and β-^{32}P GTP in isolated nuclei 14 hours post infection. Labeled RNA was isolated by phenolextraction and hybridized to ad2 DNA on filters. Hybridized RNA was eluted, digested with RNase T2 and subjected to two-dimensional analysis by electrophoresis and chromatography on PEI cellulose. Autoradiographs of β-^{32}P GTP and β-^{32}P ATP labeled samples are shown in panels A and B, respectively. The separation of marker nucleoside phosphates is shown schematically in panel C.

Table 1

Distribution of radioactivity in nucleotides obtained after RNase T2 digestion of viral RNA labeled with β-^{32}P GTP or β-^{32} ATP in vitro.

	Per cent of total radioactivity detected in				
	pppGp	ppGp	pppAp	pNp[a)]	Np
β-^{32}P GTP labeled RNA	85	15			
β-^{32}P ATP labeled RNA	5	-	17	67	11

a) Analysis of labeled pAp with nuclease P1 indicates that the label is confined to the 5' phosphate of the 3',5' mononucleosidediphosphate.

Sensitivity to α-amanitin

The three types of RNA polymerases in eukaryotic cells can be distinguished on the basis of their differential sensitivity to α-amanitin. In order to tentatively identify the RNA polymerase(s) responsible for incorporation of β-^{32}P triphosphates into RNA in isolated nuclei, incubations were carried out for 2 minutes in the presence of different concentrations of α-amanitin prior to the addition of ^{3}H-UTP and β-^{32}P labeled ATP or GTP to the incubation mixture. RNA was extracted after incubation for 45 minutes and hybridized to nitrocellulose filters with 40 µg of ad2 DNA. Table 2 shows that at low concentrations of the drug (1 µg) 40-60 % of the incorporation of ^{3}H-UTP into total RNA and around 80 % of ^{3}H-UTP incorporation into viral RNA was inhibited, whereas there was no significant decrease in the incorporation of β-^{32}P triphosphates into total or viral RNA. At 100 µg/ml of α-amanitin, around 75 % of the ^{3}H-UTP incorporation into total RNA and 95 % of the ^{3}H-UTP incorporation in viral RNA was inhibited. However, at this concentration of α-amanitin β-^{32}P GTP incorporation into viral RNA was reduced by 62 % whereas the β-^{32}P ATP incorporation was not inhibited significantly. The selective inhibition of β-^{32}P GTP incorporation at high concentrations of α-amanitin, taken together with the fact that this labeled precursor is exclusively incorporated into 5' terminal pppGp and ppGp, indicate that only initiation with RNA polymerase III can be detected in isolated nuclei with β-^{32}P GTP. From the experiments described above and those summarized in Table 1 it is impossible to draw any conclusions about chains initiated with ATP since 80 % of the label is found in structures other than nucleoside tetraphosphates.

Identification of initiated viral RNA transcripts in isolated nuclei

The number of unique viral RNA species initiated in isolated nuclei

Table 2

α-amanitin inhibition of ^{3}H-UTP or β-^{32}P nucleoside triphosphate incorporation into RNA in isolated nuclei

	Total radioactivity in RNA cpm x 10^{-3}		Hybridized radioactivity cpm x 10^{-3} a)	
	^{3}H	^{32}P	^{3}H	^{32}P
β-^{32}P GTP labeled RNA				
No drug	93.5	38.5	21.6	2.1
1 µg/ml	37.4	36.4	2.5	2.2
100 µg/ml	24.3	10.6	1.0	0.8
β-^{32}P ATP labeled RNA				
No drug	59.8	10.2	11.1	0.7
1 µg/ml α-amanitin	27.9	10.8	3.1	0.9
100 µg/ml α-amanitin	8.4	8.8	0.7	0.6

a) Nuclei isolated from 5 x 10^{5} cells at 14 hours post infection were incubated with or without α-amanitin for 2 min prior to the addition of ^{3}H-UTP or β-^{32}P GTP or ATP. RNA was hybridized for 40 hours to filters with 40 µg ad2 DNA.

can be determined in several ways. One approach is to select the viral β-^{32}P GTP labeled RNA by hybridization to filters with viral DNA and digest the eluted RNA with pancreatic RNase to generate oligonucleotides, which are specifically labeled at their 5' termini and they can be identified by two dimensional separation. Such an analysis of viral β-^{32}P GTP labeled RNA is shown in Fig. 2B. The autoradiogram demonstrates the presence of a trinucleotide and a tetranucleotide in viral RNA whereas unselected RNA contains in addition two dinucleotides (Fig. 2A). Elution and redigestion with nuclease P1 showed that the label in each of the virus specific oligonucleotides was exclusively in ppG and pppG (data not shown). A similar experiment was carried out with β-^{32}P ATP labeled unselected RNA which was digested with RNase T1 to generate oligonucleotides. The fingerprint (Fig. 2C) showed a complex pattern of oligonucleotides, as would be expected if most of the label was incorporated randomly into 5' monophosphorylated termini in RNA. The pancreatic or T1 RNase fingerprints of RNA label-

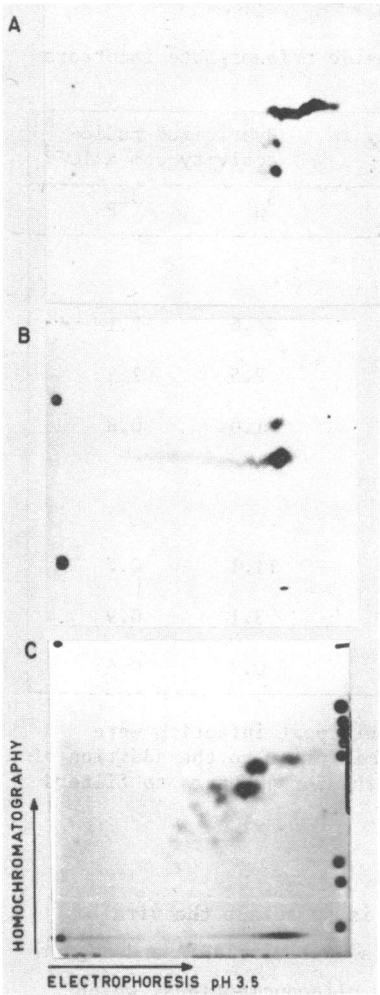

Figure 2. Pancreatic and T1 RNase fingerprints of RNA labeled with β-^{32}P GTP and β-^{32}P ATP, respectively. Labeled RNA from nuclei isolated 14 hours post infection was extracted with phenol. Viral RNA was purified by hybridization and eluted from filters prior to RNase digestion and two-dimensional analysis on DEAE thin layer. Panel A: Pancreatic RNase fingerprint of total β-^{32}P GTP labeled RNA; panel B: pancreatic RNase fingerpring of β-^{32}P GTP labeled viral RNA; pancel C: RNase T1 fingerprint of total β-^{32}P ATP labeled RNA.

ed with β-^{32}P GTP or ATP were identical with and without 1 µg/ml of α-amanitin during transcription in isolated nuclei (data not shown). The results therefore indicate that all initiated RNA molecules in isolated nuclei are products of RNA polymerase III.

In order to identify whether the 5' labeled oligonucleotides of the fingerprints correspond to known species of viral RNA, synthesis was carried out in isolated nuclei in the presence of β-^{32}P GTP or β-^{32}P ATP. RNA was extracted and fractionated by electrophoresis in 12 % polyacrylamide gels in 7 M urea. The electropherogram (Fig. 3A) of β-^{32}P GTP labeled RNA reveals ^{32}P labeled peaks in the position of viral 5.5S and 5.2S RNA as well as in the position of cellular 5.0S RNA together with

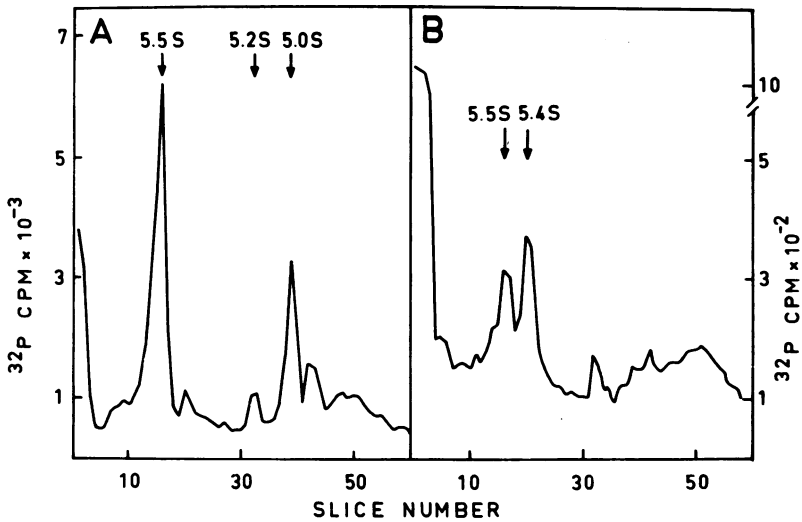

Figure 3. Polyacrylamide gel analysis of 5' terminally labeled RNA. Nuclei were incubated with β-^{32}P GTP or β-^{32}P ATP. The RNA was phenol-extracted and electrophoresed on 12 % polyacrylamide gels containing 7 M urea. The gels were sliced and the radioactivity determined. ^{32}P-labeled low molecular weight RNA from infected cells was analyzed in a parallel gel as marker. Panel A: β-^{32}P GTP labeled RNA; panel B: β-^{32}P ATP labeled RNA.

other minor species. RNA from the peaks of 5.5, 5.2 and 5.0S RNA was eluted from the gel slices and digested with pancreatic RNase. The chain lengths of the ^{32}P labeled oligonucleotides were determined by homochromatography in one dimension. Fig. 4A shows that the trinucleotide in virus specific RNA is derived from the 5.2S and the tetranucleotide from the 5.5S species. The digestion of 5.0S RNA generated a dinucleotide. Similar analysis of other regions of the gel revealed additional dinucleotides. However, it was not possible to correlate these two single RNA species. The results are in agreement with the published sequences for viral 5.5S RNA (11) and cellular 5.0S RNA (17) which predict 5' terminal (pp)pGpGpGpCp and (pp)pGpUp in pancreatic RNase fingerprints of 5.5S and 5.0S RNA respectively.

RNA labeled with β-^{32}P ATP showed a different pattern in polyacrylamide gels. Most of the radioactivity remained at the top of the gel (Fig. 3B). However, discrete peaks of radioactivity corresponding to 5.5S and 5.4S are present. It has not been possible to demonstrate which of these RNA species are virus coded since only a small fraction of the ^{32}P

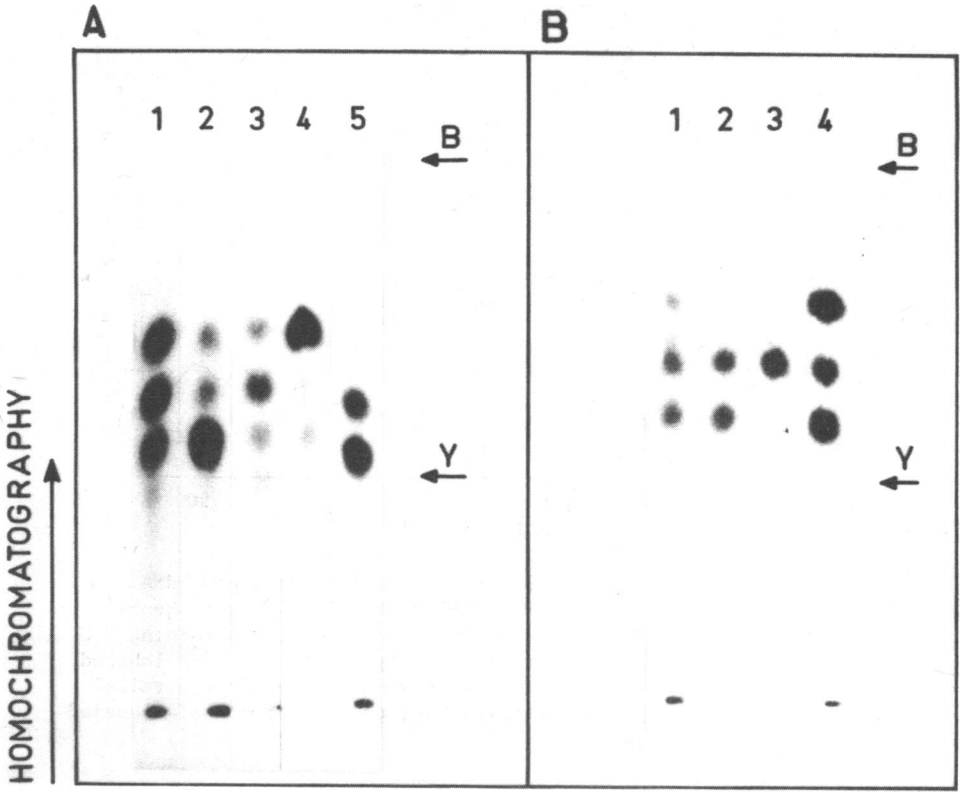

Figure 4. Pancreatic RNase oligonucleotides of 5' terminally labeled low molecular weight RNA. β-^{32}P GTP and β-^{32}P ATP labeled RNA was eluted from the gels in Figure 3, digested with pancreatic RNase and subjected to one-dimensional homochromatography on DEAE thin layers. Panel A. β-^{32}P GTP labeled RNA: 1: total RNA; 2: 5.5S RNA; 3: 5.2S RNA; 4: 5.0S RNA; 5: total adenovirus specific RNA. Panel B: β-^{32}P ATP labeled RNA: 1: total RNA; 2: 5.5S RNA; 3: 5.4S RNA; 4: Marker β-^{32}P GTP labeled RNA.

labeled RNA entered ghe gel. RNA from the top of the gel and from the peaks was eluted as indicated in Fig. 3B and the nucleotide composition was determined by RNase T2 digestion and subsequent electrophoresis and chromatography. All labeled nucleotides which previously had been identified in ^{32}P-ATP labeled RNA (Fig. 1B) were present in the high molecular weight RNA while the 5.5S RNA contained labeled pppAp and pppGp and the 5.4S RNA contained pppAp (data not shown).

It has recently been shown that two forms of viral 5.5S RNA exist in ad2 infected cells, one starting with pppGpGpGpCp (11) and one starting with pppApGpCp (1). The chain length of the oligonucleotides generated by pancreatic RNase digestion of β-^{32}P ATP labeled 5.5S and 5.4S RNA molecules

synthesized in isolated nuclei was therefore determined. The 5.5S RNA contains both a ^{32}P-labeled tri- and a tetranucleotide and the 5.4S RNA only a trinucleotide (Fig. 4B). These oligonucleotides were eluted from the thin layer plates and it was shown by redigestion with nuclease P1 that the trinucleotide contains labeled (p)ppA and the tetranucleotide labeled pppG. This suggests that the viral 5.5S RNA species which start with pppA also initiates in isolated nuclei.

Frequency of initiation of transcription with RNA polymerase III in isolated nuclei

Since no evidence for initiation of transcription of RNA polymerase II was demonstrated in isolated nuclei, it was possible to estimate the efficiency of initiation for RNA polymerase III in isolated nuclei by a simple method. The approach chosen was to incubate nuclei from ad2 infected cells with β-^{32}P GTP and ^{3}H-UTP for 30 minutes. The purified RNA was fractionated on denaturing polyacrylamide gels and the ^{32}P and ^{3}H radioactivity in 5.5S and 5.0S RNA determined. From the known specific radioactivities of the nucleosidetriphosphates and the base compositions of 5.5S and 5.0S RNA it was estimated that around 150 molecules of 5.5S or 5.0S RNA initiated per minute and nucleus (Table 3), whereas in total 400-600 molecules terminate during the same time period. These results suggest that initiation is rate limiting in vitro and only 30 % of the viral 5.5S molecules completed have initiated in vitro. Only 20 % of the cellular 5.0S RNA molecules completed are initiated in vitro.

Table 3

Frequency of initiation of transcription with RNA polymerase III in isolated nuclei

	Internal incorporation rate[a] Nucleotide x 10^{-3}/min/nucleus	5' terminal Nucleotide x 10^{-3}/ /min/nucleus	Number of RNA[b] molecules initiated and terminated in vitro per minute and nucleus	Number of RNA[c] molecules terminated but not initiated in vitro per minute and nucleus
5.5S RNA	53	23	147	396
5.0S RNA	48	20	167	630

a) estimated from incorporation of ^{3}H-UTP

b) estimated from incorporation of β-^{32}P GTP

c) the number of molecules initiated in vivo but terminated in vitro was estimated assuming that 50 % of the average molecule was transcribed in vivo and the other half in vitro.

Nuclei from ad2 infected cells were incubated with β-^{32}P GTP and ^{3}H-UTP for 30 minutes. The fraction of 5.5S and 5.2S RNA molecules initiated in vitro was established after polyacrylamide gel electrophoresis as described in the text.

Location on the adenovirus genome of genes for RNA molecules initiated and transcribed in isolated nuclei

The location of β-^{32}P GTP labeled RNA sequences in the adenovirus genome was investigated by hybridization between β-^{32}P labeled RNA and viral restriction enzyme fragments immobilized on nitrocellulose strips by the method of Southern (14). Fig. 5 shows that β-^{32}P GTP labeled RNA hybridizes to fragments BamHI-B and -D, fragments BalI-B and -M, and fragment SmaI-B. The same pattern of hybridization was obtained with a mixture of 5.5S and 5.2S RNA labeled in vivo with ^{32}P.

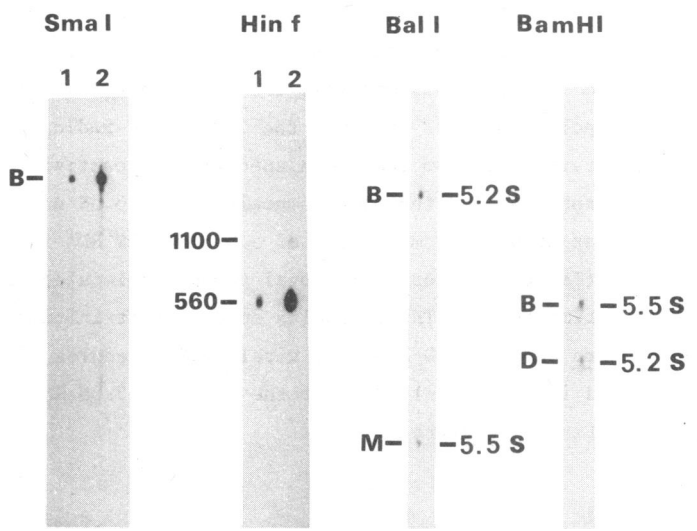

Figure 5. Hybridization of β-^{32}P GTP labeled RNA to restriction enzyme fragments of ad2 DNA. Labeled RNA was extracted from isolated nuclei and hybridized to ad2 DNA restriction enzyme fragments immobilized on nitrocellulose strips. 1. Hybridization with β-^{32}P GTP labeled RNA. 2. Hybridization with in vivo $^{32}PO_4$ labeled 5.5S and 5.2S RNA (SmaI and Hinf). For the BalI and BamHI fragments only hybridization with β-^{32}P GTP labeled RNA is shown.

Hybridizations were also carried out with fragments generated with endonuclease Hinf, which cleaves ad2 DNA into a large number of small fragments. Recent studies (18) have shown that the entire 5.5S RNA and the 5' terminal part of 5.2S RNA hybridizes to a 560 nucleotide long fragment, whereas the 3' terminal end of 5.2S RNA hybridizes to a fragment approximately 1100 nucleotides long. RNA labeled with β-^{32}P GTP in isolated

nuclei hybridizes exclusively to the 560 nucleotide long fragment which is expected since the 5' termini of both 5.5 and 5.2S RNA are located within this fragment. Hybridization of RNA labeled with β-^{32}P GTP in isolated nuclei in the presence of 1 μg/ml of α-amanitin showed an identical pattern of hybridization (not shown).

DISCUSSION

In vitro nuclei isolated late in adenovirus infection incorporate β-^{32}P GTP into 5' termini of RNA. The label is confined to the β-position of the nucleotide (p)ppGp in RNA and incorporation is inhibited at high concentrations of α-amanitin, suggesting that incorporation reflects initiation of transcription. The labeling of RNA with β-^{32}P ATP in isolated nuclei is, in contrast, not specific for initiation of transcription. Only 17 % of the label in viral RNA is recovered in (p)ppAp. Most of the label is recovered in the 5' phosphate of 3', 5' mononucleosidediphosphates, mainly pAp. The latter incorporation is not inhibited by high concentrations of α-amanitin (Table 2). Labeling of the 5' phosphates of mononucleosidediphosphates may occur through pyrophosphate exchange which generates γ-^{32}P ATP from the β-labeled precursor. The γ-phosphate could then be utilized for phosphorylation of 5' hydroxyl (19) in RNA. Some label was also observed in 3' nucleosidemonophosphates with β-^{32}P ATP which could arise by phosphorylation of ribonucleotides either *in vitro* or by uptake of $^{32}PO_4$ by a few unbroken cells in the nuclear preparations.

The β-^{32}P GTP initiated viral RNA was characterized by hybridization and pancreatic RNase fingerprint analysis. Only a trinucleotide and a tetranucleotide were recovered in viral RNA, while unselected RNA contained two additional dinucleotides. These four oligonucleotides were also present in RNA when the nuclei were incubated with 1 μg/ml of α-amanitin (not shown), indicating that only RNA polymerase III products were initiated. β-^{32}P GTP labeled viral 5.5S and 5.2S RNA and cellular 5.0S RNA were isolated after gel electrophoresis and their labeled pancreatic RNase oligonucleotides analyzed. The correct 5' terminal oligonucleotides were recovered for 5.5S RNA (11), 5.2S RNA (B. Vennström, unpublished) and 5.0S RNA (17). The β-^{32}P GTP labeled RNA furthermore showed a hybridization pattern to ad2 DNA restriction enzyme fragments which would be predicted from the known locations of the 5' termini of 5.5S and 5.2S RNA. These results suggest that the initiation with RNA polymerase III in isolated nuclei is specifically measured with β-^{32}P GTP, although the rate of initia-

tion is low (Table 3).

The complex T1 RNase fingerprint of β-^{32}P ATP labeled unselected RNA indicates that the labeling is not confined to RNA termini. Virus specific RNA was not analyzed by fingerprinting because of the low yields of labeled viral RNA. However, gel electrophoresis of the low molecular weight RNA revealed both a 5.5S and a 5.4S species with the same migration rate as the two in vivo species of 5.5S RNA (1). Analysis of oligonucleotides generated by pancreatic RNase and T2 RNase digestion of these two species labeled with β-^{32}P ATP yielded a tetranucleotide with a 5' terminal pppGp from the 5.5S peak and a tetranucleotide and a trinucleotide with 5' terminal pppGp and pppAp respectively from the 5.4S peak. These results suggest that β-^{32}P ATP may also initiate transcription faithfully since the same two species of viral 5.5S RNA were observed in vivo as reported in an accompanying paper (1).

Attempts were also made to detect initiation with RNA polymerase II in isolated nuclei by comparing the hybridization patterns of β-^{32}P ATP labeled RNA with and without 1 µg/ml of α-amanitin. The two patterns were identical, indicating that initiation with polymerase II was, if it occurs, below the level of detection in isolated nuclei.

Gilboa et al. (20) also used β-^{32}P triphosphates to detect initiation of transcription in isolated nuclei from Friend cells. In their system both β-^{32}P GTP and β-^{32}P ATP gave specific labeling of the 5' termini of RNA. The reason for the discrepancy between their and our results is unclear, but may be due to the source of cells.

In summary, isolated nuclei from adenovirus infected HeLa cells are initiating transcription of RNA polymerase III products but initiation of polymerase II products cannot be detected. The stable viral 5.5S and 5.2S RNA species are faithfully initiated in isolated nuclei.

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