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Two initiation sites for adenovirus 5.5S RNA

Björn Vennström, Ulf Pettersson and Lennart Philipson

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

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ABSTRACT

Adenovirus specific 5.5S RNA is heterogeneous at its 5' terminus. Complete pancreatic RNase digests of the RNA reveal a 5' terminal oligonucleotide (pp)pApGpCp in addition to the major 5' terminal (pp)pGpGpGpCp (1). Both 5' termini are detected early as well as late after adenovirus infection. In isolated nuclei, α-amanitin inhibits all 5.5S RNA transcription at a concentration of 200 µg/ml, indicating that both initiation sites are recognized by RNA polymerase III.

INTRODUCTION

In cells infected with adenovirus type 2 (ad2), two virus specific low molecular weight RNA species of unknown function, 5.5S and 5.2S RNA, are transcribed (1,2,3). Both species are produced early and late after infection, are transcribed by RNA polymerase III in isolated nuclei (3,4,5,6) and have been reported to initiate with guanosinetetraphosphate (3,4). The nucleotide sequence of the 5.5S RNA, which contains 157-160 nucleotides, has been determined (1, Weissmann, S.M., personal communication). The genes for both 5.5S and 5.2S RNA map in a region of the viral genome which is located approximately 30 % from the left hand end (2,3,7). The spacer region between the 5.5S RNA and the 5.2S RNA has been estimated to be about 75 nucleotides long (8).

In this communication an additional 5'-terminal sequence of 5.5S RNA, starting with adenosinetetraphosphate, is identified. Both the adenosine and the guanosine initiated 5.5S RNA molecules are synthesized by RNA polymerase III.

MATERIALS AND METHODS

Infection and labeling of cells

HeLa cells were grown in suspension cultures in Eagle's spinner medium or as monolayers in Eagle's basal medium. The cells were infected with ad2

as described previously (9). For labeling with $^{32}PO_4$, cells were washed twice with phosphate free medium supplemented with 50 mM HEPES and were incubated with 100-300 µC/ml of $^{32}PO_4$ in the phosphate free medium with 7 % dialyzed calf serum. Cytoplasmic extracts were prepared (10) and phenol extracted (11). Low molecular weight RNA was isolated by sucrose gradient centrifugation (7). Nuclei for in vitro transcription were isolated from infected cells by Dounce homogenization of hypotonically swollen cells and incubated in the presence of α-^{32}P GTP as described previously (9). The incubation mixtures were phenol-extracted and DNA was eliminated by two digestions with DNase I (100 µg/ml) for 20 min at 37°C. After each digestion the RNA was extracted with phenol and ethanol precipitated (9). The excess of labeled nucleoside triphosphates was removed by gel filtration on Sephadex G50 (12) or by cellulose chromatography (13).

Acrylamide gel electrophoresis

Electrophoresis in 10 % polyacrylamide gels at 4°C was carried out as described previously (7,8). Electrophoresis in polyacrylamide gels containing 7 M urea (8,14) was performed at 20°C with a voltage of 5 V/cm .

Nucleic acid hybridization

Preparative hybridization between labeled RNA and ad2 DNA immobilized on nitrocellulose filters was carried out as described previously (9). RNA was eluted from the filters by boiling for 2 minutes in 10 mM Tris-HCl pH 7.9, 1 mM EDTA and 0.2 % SDS. The eluted RNA was extracted with phenol and precipitated with ethanol. To eliminate DNA which had detached from the filters, the RNA was treated with DNase I, phenol extracted and ethanol precipitated twice. The recovery of filter bound RNA was around 75 %.

Nucleotide analysis

Pancreatic RNase digestions were made in 100 mM NH_4CO_3, 1 mM EDTA. RNase T1 was used in 10 mM Tris-HCl, 1 mM EDTA pH 7.9. Digestions were carried out for 30 minutes at 37°C at an enzyme substrate ratio of 1/10 (w/w). RNase T2 (Sankyo) digestions were made in 50 mM NaAc, 1 mM EDTA pH 4.5 for 3 hours at 37°C at an enzyme substrate ratio of 1/3 (u/w). Mixed digestions with T1, T2 and pancreatic RNase were performed as indicated. Separation of (oligo)nucleotides generated by T2 or pancreatic RNase was performed by high voltage electrophoresis at pH 3.5 on cellulose acetate in the first dimension and homochromatography on DEAE or PEI thin layer plates in the second dimension (15). For RNase T1 fingerprints,electrophoresis on DEAE cellulose paper in 7 % formic acid was used as the second dimension (16). Thin layer chromatograms on PEI plates were developed stepwise with water to the origin, 0.3 M

LiCl in 1 M HCOOH to 10 cm and 1.5 M LiCl in 1 M HCOOH to 20 cm (19).

The resulting chromatograms were analyzed by autoradiography, using Kodak No Screen film at room temperature or prefogged Kodak RP54 film and intensifying screens at -70°C (R. Laskey, personal communication).

RESULTS

Initiating nucleotides in viral 5.5S RNA

Low molecular weight RNA was extracted from HeLa cells labeled with $^{32}PO_4$ from 14 to 18 hours after infection with ad2 and fractionated by sucrose gradient centrifugation. RNA sedimenting at 4-7S was subjected to electrophoresis in 10 % nondenaturing polyacrylamide gels as shown in Fig. 1A. The 5.5S RNA was eluted from the gel and hybridized to 60 µg of ad2 DNA on nitrocellulose filters. Hybridized RNA was eluted, digested with RNase T2 and analyzed by two dimensional electrophoresis and homochromatography. As shown in Fig. 2,virus specific 5.5S RNA contains both 5' terminal (pp)pAp and (pp)pGp. The results suggest that 5.5S RNA can initiate both with guanosine and adenosine tetraphosphate.

The relative amounts of adenosine and guanosine initiated 5.5S RNA were estimated by determination of radioactivity in the spots corresponding to 5' terminal nucleotides. Selection of RNA on ad2 DNA was omitted since

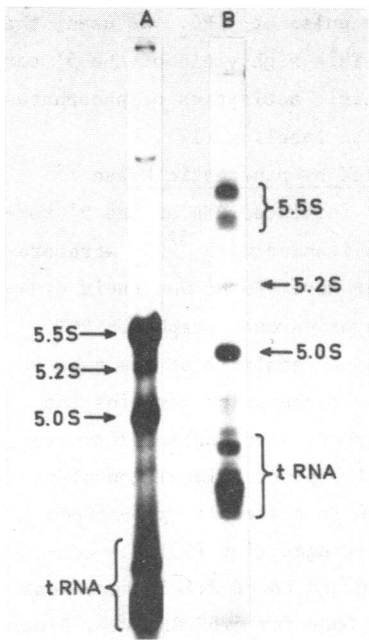

Figure 1. Polyacrylamide gel electrophoresis of low molecular weight RNA from adenovirus infected cells. A: electrophoresis in a 10% polyacrylamide gel under nondenaturing conditions; B: electrophoresis in a 12 % polyacrylamide gel containing 7 M urea.

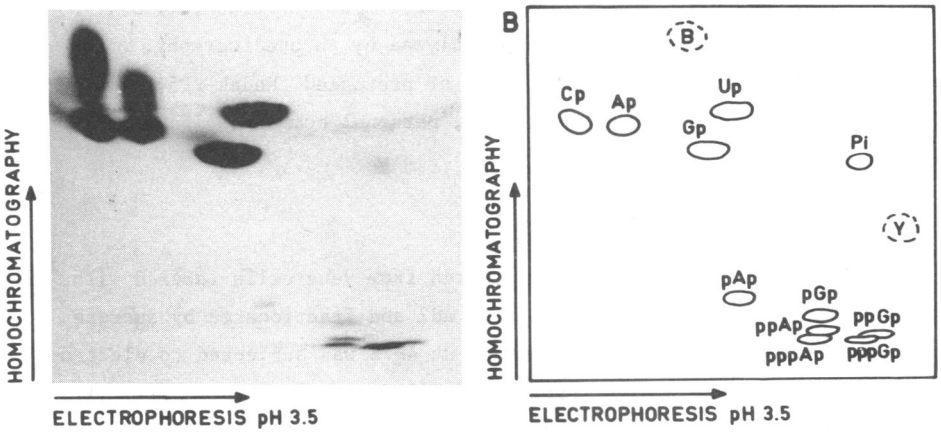

Figure 2. RNase T2 finger print of viral 5.5S RNA. The RNA was extracted from a 10 % nondenaturing gel and annealed to nitrocellulose filters carrying ad2 DNA. Hybridized RNA was eluted, digested with RNase T2 and analyzed by electrophoresis and homochromatography on PEI plates in two dimensions. A: Autoradiograph of the chromatogram; B: Schematic diagram of marker nucleotides.

the contamination with cellular RNA is negligible (Figs. 3 and 6). Around 25 % of the 5.5S RNA initiates with (pp)pAp and 75 % with (pp)pGp (not shown). Ten per cent of the 5.5S RNA contains monophosphorylated, 40 % diphosphorylated and 50 % triphosphorylated 5' termini. When a 4 hour pulse of $^{32}PO_4$ was used, the molar yield of 5' termini was 1.25 (not shown). This high yield of the 5' terminal nucleotides is probably due to higher specific activities of phosphates in the γ and β than in the α positions after pulse labeling (17).

Analysis of 5' terminal oligonucleotides generated by pancreatic RNase

To establish the sequence of the adenosine initiated RNA at the 5' terminus, pancreatic RNase fingerprints of 5.5S RNA labeled with $^{32}PO_4$ were produced as shown in Fig. 3. A large number of spots were eluted and their nucleotide composition was determined by thin layer chromatography on PEI cellulose after RNase T2 digestion. Fig. 4 shows the analysis of the two spots of interest; spot A in the pancreatic RNase fingerprint contains the 5' terminal (pp)pAp, Gp and Cp. The two monophosphorylated nucleosides were recovered in a 1:1 molar ratio. The sequence of the pancreatic oligonucleotide originating with triphosphorylated adenosine is therefore (pp)pApGpCp. Fig. 4 also shows that spot B in the pancreatic fingerprint (Fig. 3) contains (pp)pGp, Gp and Cp with a molar ratio of Gp and Cp of 2:1 in agreement with the reported 5' terminal sequence (pp)pGpGpGpCp for 5.5S RNA (1). Since

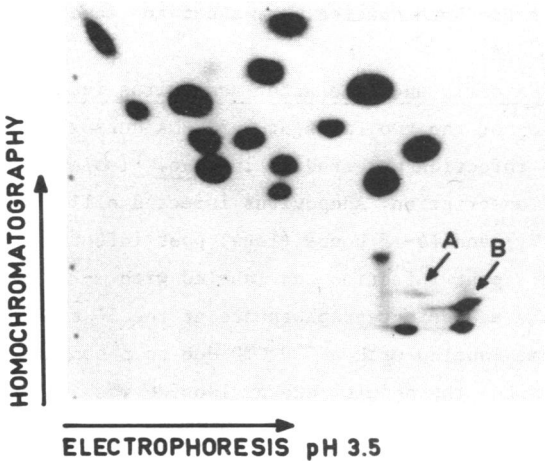

Figure 3. Pancreatic RNase fingerprint of 5.5S RNA. The RNA was isolated after polyacrylamide gel electrophoresis, digested with pancreatic RNase and analyzed by electrophoresis and homochromatography on PEI plates in two dimensions. Arrows A and B indicate the 5' terminal oligonucleotides.

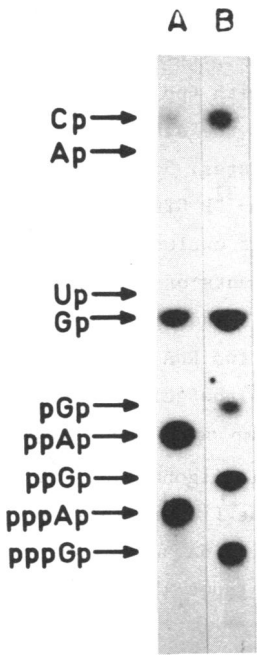

Figure 4. RNase T2 analysis of the 5' terminal oligonucleotides generated by pancreatic RNase. Spots A and B in Fig. 3 were eluted, redigested with a mixture of T1, T2 and pancreatic RNase and analyzed by chromatography on PEI plates. Lane A: analysis of spot A in Fig. 3; Lane B: analysis of spot B in Fig. 3.

the 5' terminal sequence AGC is not present until position 153 in the revised sequence of 5.5S RNA (Weissman, S.M., personal communication) the results infer that the AGC start is located immediately to the left of the major

start for 5.5S RNA, since both species have about the same length on non-denaturing gels (Fig. 1A).

5' termini in 5.5S RNA early and late after adenovirus infection

The transcription of the two forms of 5.5S RNA during the early and late phases of adenovirus infection was studied in vivo. Isolated nuclei were also used to study late transcription. Adenovirus infected cells were labeled with $^{32}PO_4$ 2-6 hours (early) and 14-18 hours (late) post infection. Isolated nuclei were prepared 14 hours post infection and labeled with α-^{32}P GTP for 45 min. In the latter case the adenosinetetraphosphate at the 5' termini of 5.5S RNA was expected to become labeled with α-^{32}P GTP due to phosphate transfer after RNase T2 digestion, since the penultimate nucleoside was guanosine in the species initiating with adenosine (Fig. 4). Labeled RNA was purified and electrophoresed on 12 % polyacrylamide gels containing 7 M urea as shown in Fig. 1 B. In these gels 5.5S RNA separates into a slow and a fast migrating species. The two components were extracted, digested with RNase T2 and subjected to thin layer chromatography on PEI cellulose. Fig. 5 shows that, early after ad2 infection, the slowly migrating component of 5.5S RNA contains only (pp)pGp, while the faster component contains both (pp)pAp and (pp)pGp. Both components labeled late after infection contain (pp)pAp and (pp)pGp, although the fast component is enriched for molecules initiating with (pp)pAp. In other experiments involving two-dimensional gel electrophoresis the slow species contained only (pp)pGp and the fast species both tetraphosphates. 5.5S RNA molecules synthesized in isolated nuclei in the presence of α-^{32}P GTP also separate into two components. The slowly migrating species contains exclusively (pp)pGp and the fast migrating species (pp)pAp and only small amounts of (pp)pGp. These results suggest that the adenosine initiated molecules are shorter than the ones initiating with guanosine or alternatively that the two RNA species differ in secondary structure even in the presence of 7M urea in the gels.

To differentiate between these alternatives both components were subjected to RNase T1 fingerprinting, and the amount of 3' terminal oligonucleotides was quantitated relative to internal oligonucleotides. The fingerprints of the two components are virtually identical as shown in Fig. 6A and B. The arrows indicate the 3' terminal oligonucleotides CUCCUU (spot 45) and CUCCUUU (spot X_1) (1, Mathews, M., personal communication, Weissman, S.M., personal communication). Spot 45 was present in a molar ratio of 0.5 and spot X1 in a ratio of 0.25 in both species. These results indicate that the two 5.5S RNA species differ only in their 5' terminal sequence and that they have a similar extent of heterogeneity in their 3' termini. It is therefore

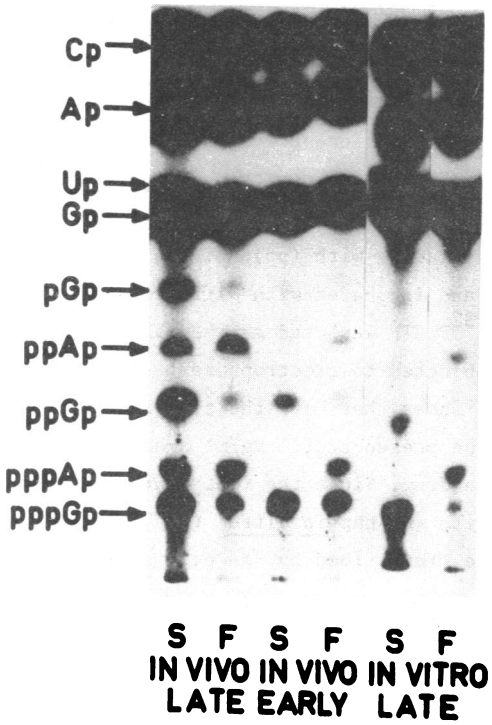

Figure 5. RNase T2 analysis of 5.5S RNA labeled with $^{32}PO_4$ in vivo or α-^{32}P GTP in vitro. RNA was labeled in vivo at 2-6 hours post infection (early RNA) or at 14-18 hours post infection (late RNA). RNA was labeled in vitro by incubating isolated nuclei with α-^{32}P GTP 14 hours post infection for 45 minutes. Slow (S) and fast (F) migrating 5.5S RNA was isolated from polyacrylamide gels containing 7 M urea (Fig. 1 B) and digested with RNase T2. Analysis was carried out by PEI thin layer chromatography.

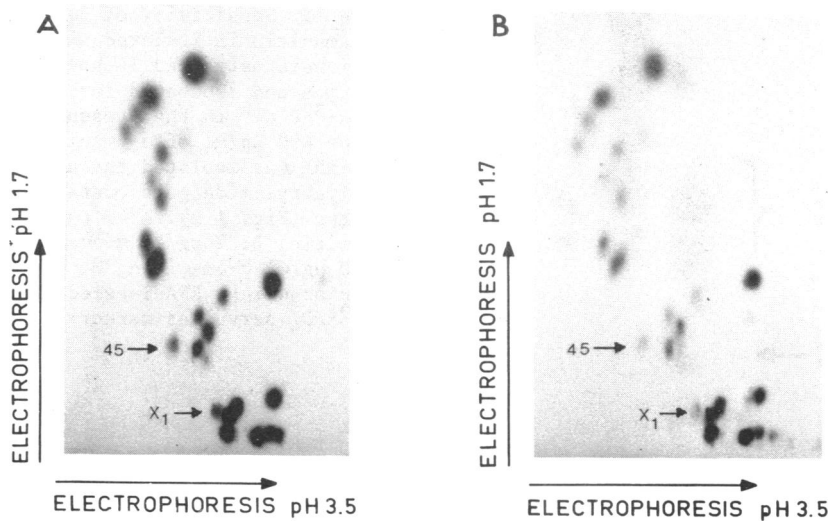

Figure 6. RNase T1 fingerprint of the two species of viral 5.5S RNA. RNA labeled in vivo with $^{32}PO_4$ was isolated after polyacrylamide gel electrophoresis as shown in Fig. 1B, digested with RNase T1 and analyzed by electrophoresis on cellulose acetate and DEAE paper. A: Slow migrating 5.5S RNA; B: fast migrating 5.5S RNA.

most likely that their different migration rates in urea-polyacrylamide gels is due to differences in secondary structure.

Effect of α-amanitin on the synthesis of the two 5.5S RNA species

The eukaryotic RNA polymerases exhibit different sensitivities to α-amanitin. Since guanosine initiated 5.5S RNA is known to be transcribed by RNA polymerase III, experiments were designed to establish whether the same polymerase transcribes the species initiating with (pp)pAp. Isolated nuclei were prepared 14 hours post infection and incubated with different concentrations of α-amanitin for 45 minutes. α-^{32}P GTP was used as the labeled precursor. Total RNA was extracted and subjected to electrophoresis in polyacrylamide gels containing 7 M urea. Fig. 7 shows that both the slow and the fast migrating species are transcribed in the presence of 1 µg/ml α-amanitin but not in the presence of 200 µg/ml of the drug. Since the fast migrating component is almost exclusively initiated with (pp)pAp in vitro (Fig. 5) these results indicate that both species are transcribed by RNA polymerase III.

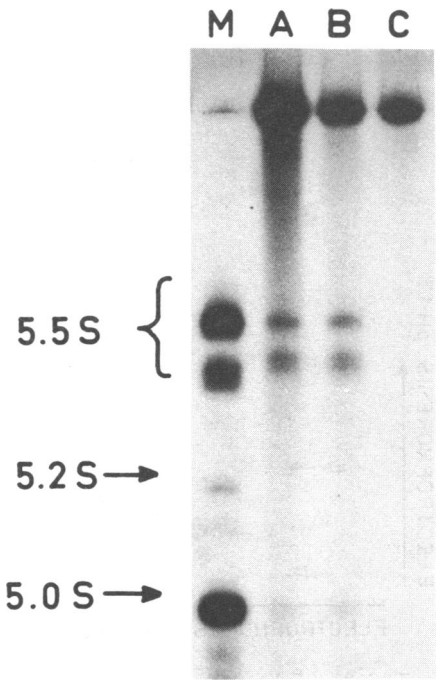

Figure 7. Sensitivity of 5.5S RNA to α-amanitin in isolated nuclei. Nuclei were harvested 14 hours post infection and incubated for 45 min with α-^{32}P GTP in the presence of 0, 2 or 200 µg/ml of α-amanitin. Total RNA was isolated and analyzed on polyacrylamide gels containing 7 M urea (Fig. 1 B). A: 0 µg/ml α-amanitin; B: 2 µg/ml α-amanitin; C: 200 µg/ml α-amanitin; M: Low molecular weight RNA labeled in vivo with $^{32}PO_4$ served as markers.

DISCUSSION

Adenovirus specific 5.5S RNA consists of two species with different 5' termini both of which appear to be transcribed by RNA polymerase III. The majority, 75 %,of the molecules initiate with pppGp and the remaining 25 %, initiate with pppAp. In both cases,the 5' terminal nucleotide shows a variable degree of phosphorylation; 50 % of the termini are triphosphorylated, 40 % diphosphorylated and 10 % monophosphorylated. Cellular 5.0S RNA has also a variable extent of phosphorylation in its 5' terminus (18).

Analyses of oligonucleotides generated by pancreatic RNase digestion of 5.5S RNA show that the 5' terminal sequence of the adenosine initiated RNA is (pp)pApGpCp. We conclude that the start of this RNA species is located leftwards of the (pp)pGp-start because of the following reasons: Within the revised sequence for 5.5S RNA (Weissman, S.M.,personal communication) the sequence AGC does not occur until position 153. Since both species of 5.5S RNA are similar in length and have nearly identical T1 fingerprints we must conclude that the (pp)pAp-start is located leftwards on the viral genome. It is interesting to note that within the DNA sequence which proceeds the gene for 5.5S RNA the sequence AGC has been found to be located immediately to the left of the sequence GGGC which is known to be the 5' terminal sequence of the major species of 5.5S RNA (Weissman, S.M., personal communication, Mathews, M.B., personal communication). Initiation of the adenosine initiated RNA may therefore occur 3 nucleotides leftwards of the position where the major guanosine initiated RNA starts.

The finding that the adenosine initiated species migrates faster in 7 M urea containing polyacrylamide gels is unexpected and could suggest that the adenosine starting 5.5S is altered at its 3' terminus. We find, however, that the two forms of 5.5S RNA have the same extent of 3' terminal heterogeneity since the 3' terminal oligonucleotides CUCCUU and CUCCUUU are present in the same submolar amounts. Thus we are forced to conclude that the different migration rates in 7 M urea polyacrylamide gels reflect conformational differences rather than difference in chain length in this particular case. This is possible since 7 M urea is insufficient to completely denature RNA and similar anomalous mobilities have previously been observed for 5.8S host cell RNA (8).

We have presently no clue to the biological significance of the two forms of 5.5S RNA. Similar observations were recently made by Celma, Pan and Weissman (personal communication).

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