Journal of Virology, Apr. 1984, p. 255–257 Vol. 50, No. 1
0022-538X/84/040255-03$02.00/0
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NOTES

Effect of the β-γ Phosphate Bond of ATP on Synthesis of Leader RNA and mRNAs of Vesicular Stomatitis Virus

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Received 22 September 1983/Accepted 12 December 1983

RNA products synthesized in vitro by vesicular stomatitis virus with normal nucleotides or imido analogs were compared by polyacrylamide gel electrophoresis. When imido ATP, which has a nonhydrolyzable β-γ bond, was substituted for ATP, leader RNA and DI particle product were synthesized, but appreciable mRNA synthesis was not detected.

In vitro studies with vesicular stomatitis virus have demonstrated that efficient transcription of the viral genome requires much higher concentrations of ATP than of the other three nucleotides; the reported K_m for ATP is ca. 500 μM, compared with 22 μM for GTP and 33 μM for CTP and UTP (13). Although the exact requirement for the high concentration of ATP has not been defined, an examination of vesicular stomatitis virus transcription suggests a number of steps in which ATP could be involved (1, 2). The virus-encoded polymerase transcribes the genome in a sequential and polar manner in the gene order 3′-leader-*N*-*NS*-*M*-*G*-*L*-5′. Leader RNA is a 47-nucleotide-long transcript which initiates with A and contains a high proportion of A residues but is not polyadenylated. The *N* through *L* genes code for five monocistronic mRNAs which are capped and methylated at the A residue on their 5′ end and are polyadenylated at their 3′ end. The polymerase enters at the 3′ end of the genome (6), and transcription occurs by either a stop-start or a processing mechanism (2). Since leader RNA begins with the sequence (p)ppACG (5), it has been suggested that the high K_m for ATP reflects initiation with an A residue. This interpretation is based on in vitro studies in which preincubation of virus with CTP and high concentrations of ATP allows subsequent RNA synthesis at low concentrations of ATP (13). Imido ATP, which has a nonhydrolyzable β-γ phosphate bond (10, 15), inhibits in vitro transcription as assayed by incorporation of labeled nucleotides into acid-insoluble material; if virus is preincubated with ATP and CTP, then some RNA synthesis occurs when imido ATP is substituted for ATP. From this it was concluded that cleavage of the β-γ bond of ATP was required for initiation at the 3′ end of the viral genome.

We have carried out in vitro transcription experiments with imido analogs. Figure 1 compares the solubilized standard and defective-interfering (DI) virion transcription products synthesized with ATP, imido ATP, or imido GTP. Transcription of standard virions with ATP generates leader RNA, the 5′-*N* gene oligonucleotides described previously (11), and high-molecular-weight RNA indicative of messenger synthesis. However, when imido ATP is substituted for ATP, only leader RNA and the *N* oligonucleotides appear to be synthesized. A longer exposure of the same gel shows the presence of larger transcripts which could be due to either leader-*N* read-through (4, 7, 14) or *N* mRNA synthesis from polymerase already at the promoter, but a comparison of lanes A and E shows that imido ATP preferentially inhibits

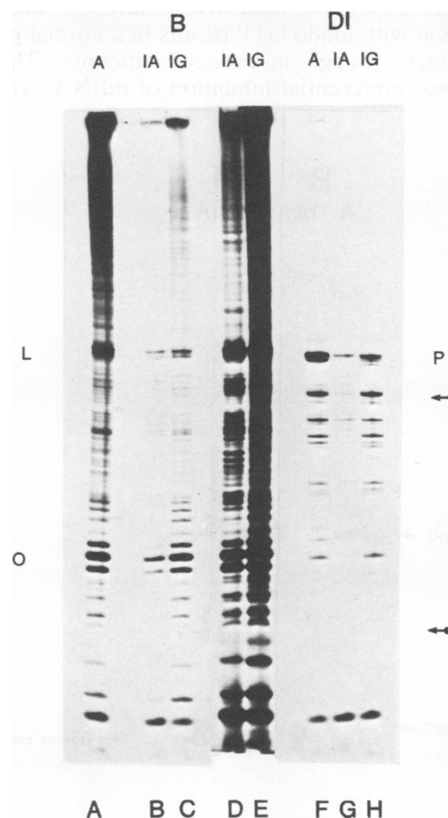

FIG. 1. Autoradiograph of a 20% polyacrylamide gel with 7 M urea showing the [α-^{32}P]UTP-labeled transcripts synthesized in a standard in vitro reaction (11) by wild-type (lanes A through E) and DI (lanes F through H) virions. RNA synthesized for 3 h at 31°C was phenol extracted, ethanol precipitated, and lyophilized. Samples were resuspended in 7 M urea with xylene cyanol and bromophenol blue before electrophoresis. Lanes D and E are the same as lanes B and C, respectively, but the autoradiograph was exposed seven times as long. Lanes A and F, 1 mM ATP; lanes B, D, and G, 1 mM imido ATP; lanes C, E, and H, 1 mM imido GTP. The location of leader RNA (L), DI product (P), the 11- to 14-nucleotide-long 5′ *N*-gene oligonucleotides (O), and the dyes (arrows) are noted.

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TABLE 1. Effect of different nucleotides on total RNA synthesis[a]

Expt	TCA-precipitable RNA (cpm) with:		
	ATP	imido ATP	imido GTP
B virions			
1	1,204,387	77,100 (6)	390,390 (32)
2	1,119,191	40,212 (4)	267,163 (24)
3	1,158,765	63,804 (6)	326,133 (28)
DI virions			
1	203,206	60,455 (30)	125,307 (62)
2	99,559	24,383 (24)	56,754 (57)
3	233,529	65,050 (28)	172,317 (74)

[a] Total RNA synthesis was determined by the amount of trichloroacetic acid (TCA)-precipitable material in each sample, with the percentage of the ATP sample for each experiment given in parentheses. Virions were incubated for 3 h in a 15-μl reaction mixture with [α-^{32}P]UTP as the labeled substrate, as described previously (11). Water (300 μl) was added to each sample, and 100 μl was placed into each of three tubes. Carrier RNA was added, and the samples were precipitated with 12.5% trichloroacetic acid and filtered. The results are given as the sum of the radioactive counts of the three aliquots minus background.

the large RNA synthesis indicative of mRNA transcription. Transcription with imido GTP results in a normal pattern of RNA products though in reduced amounts. This result indicates that preferential inhibition of mRNA synthesis is specific for the ATP analog and is not due solely to the presence of an imido group. DI virions synthesize mainly the 46-nucleotide-long DI product whether ATP, imido ATP, or imido GTP is included.

Table 1 shows the incorporation of [α-^{32}P]UTP into acid-precipitable RNA. Imido ATP causes a drastic decrease in incorporation by the standard virus which correlates with the inhibition of mRNA synthesis seen on the gel, but there is much less inhibition of incorporation by DI virions which do not synthesize mRNA. This demonstrates that the major effect of imido ATP is on mRNA synthesis. Imido GTP has less of an inhibitory effect, and the results indicate that the imido analog is used for transcription about one-third to one-half as efficiently as the normal nucleotide.

Next, we directly determined that imido ATP could initiate transcripts. An in vitro transcription reaction lacking UTP and GTP, but containing ATP and CTP, will generate large amounts of (p)ppAC representing the 5′ end of leader RNA or DI particle product (3, 6). Calf intestinal alkaline phosphatase will remove the 5′ terminal phosphates from the ATP-initiated dimer, but the imido group protects the analog-initiated dimer from phosphatase digestion, so the two A-C dimers can be distinguished by gel electrophoresis. Figure 2 indicates that imido ATP can initiate transcription

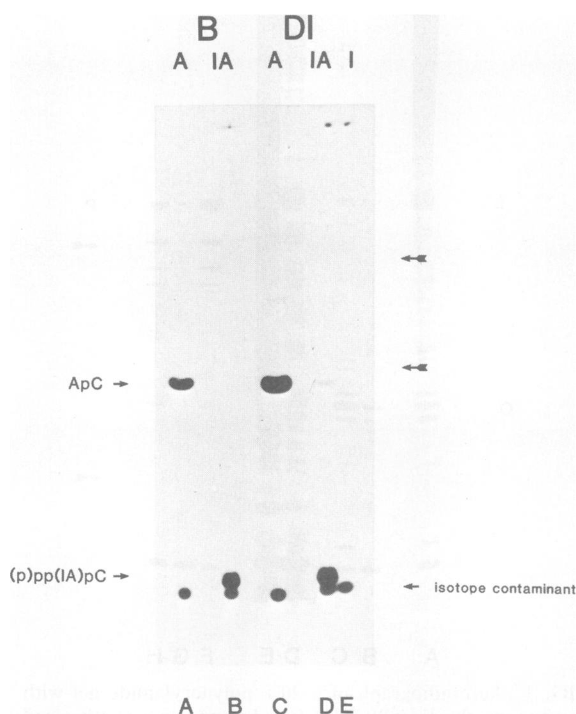

FIG. 2. Autoradiograph of a 20% polyacrylamide gel showing A-C dimers synthesized by B (lanes A and B) and DI (lanes C and D) virions in the absence of UTP and GTP (6). The [α-^{32}P]CTP-labeled products synthesized at 31°C for 3 h were phenol extracted, ethanol precipitated, and treated with calf intestinal alkaline phosphatase (0.52 U in 0.1 M Tris [pH 8.0]) for 60 min at 37°C. Urea (7 M) was added before electrophoresis on gels containing no urea. Bromophenol blue and xylene cyanol (arrows) were run in lanes with no samples. Lanes A and C, 1 mM ATP (A); lanes B and D, 1 mM imido ATP (IA); lane E, isotope control, no virus (I).

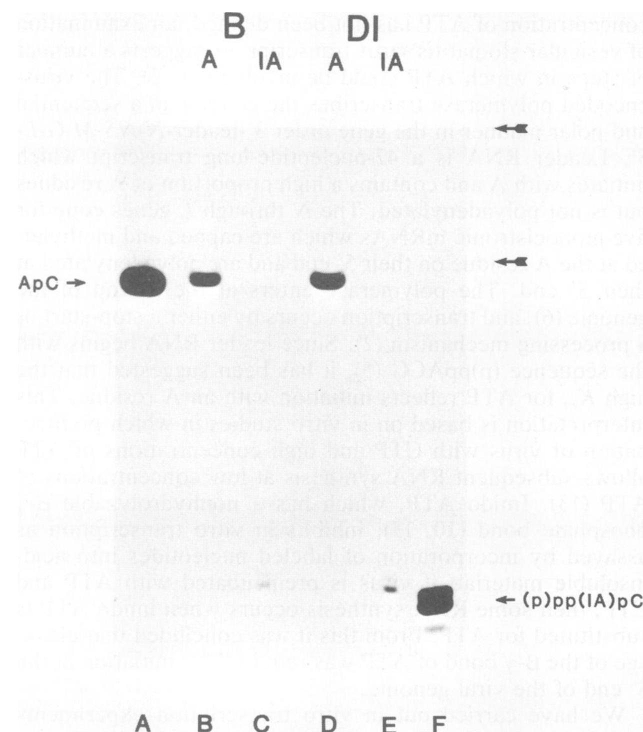

FIG. 3. Autoradiograph of a 20% polyacrylamide gel showing the 5′ initiating dimers isolated from leader RNA (lanes B and C) and DI product (lanes D and E). [α-^{32}P]CTP-labeled leader RNA and DI product synthesized in complete reactions containing ATP (A) or imido ATP (IA) were eluted from 20% gels and treated with RNase T_1 (20 U in 10 mM Tris [pH 7.6]–0.5 mM EDTA), and the products were separated on 20% gels. The 5′ initiating trimers were eluted and treated with RNase A (0.186 μg in 0.05 M Tris [pH 8.0]) followed by calf intestinal alkaline phosphatase (3.4 U in 0.1 M Tris [pH 8.0]), and the products were then reanalyzed on 20% polyacrylamide gels. The gels were identical to those in Fig. 1 except that urea was omitted. Lane A, A-C marker; lanes B and D, 1 mM ATP; lanes C and E, 1 mM imido ATP; lane F, imido A-C marker.

of leader RNA and the DI product. There is some ATP contamination in imido ATP, but it is less than 0.1% (10). Since T_1 oligonucleotides of the 5′ end of the leader RNA and DI product were also resistant to phosphatase treatment and displayed the characteristic mobility on gels, transcripts containing all four nucleotides were also initiated with imido ATP (Fig. 3).

The results presented demonstrate that both initiation and elongation of transcripts occur with imido ATP, but under our conditions a restricted set of transcripts is synthesized and mRNA synthesis is preferentially repressed. This is in apparent conflict with earlier results (13) which stated that imido ATP could elongate but not initiate transcripts. However, this conclusion was based on the drastic reduction of acid-precipitable counts, and in no case was the RNA synthesized by uninitiated cores in the presence of imido ATP ever directly examined.

There are several explanations for our results in view of the high K_m for ATP. Because leader RNA, but little mRNA, was synthesized with imido ATP, cleavage of the β-γ bond of ATP may be required for mRNA synthesis. The synthesis of the 5′-*N* oligonucleotides indicates that imido ATP can be used for internal initiations once the transcriptase is bound to the beginning of a gene. Since there are four nucleotides separating the leader RNA and *N* genes (8), if these nucleotides are not normally transcribed, ATP hydrolysis may be required to translocate the transcriptase from the end of the leader gene to the start of the *N* gene. Alternatively, the polymerase proteins NS is a phosphoprotein, and the extent of phosphorylation affects its function (9, 12). Since imido ATP is not a substrate for protein kinases, inhibition of mRNA synthesis may reflect a requirement for NS phosphorylation before mRNA synthesis. A third possible explanation is based on the prevalence of A residues as well as long stretches of the nucleotide in leader RNA and the mRNAs. Since the transcriptase does not recognize imido ATP as well as ATP, on reaching a long stretch of A residues the polymerase may have an increased chance of falling off the template. Thus, few transcriptases would reach the end of leader RNA, and random termination of any *N* gene transcripts would make them difficult to detect on gels. Polyadenylation would probably be inhibited, and if that is necessary before the next gene, *NS*, is transcribed, total incorporation would be drastically reduced. At present, we cannot distinguish among these possibilities.

We thank Karen Williamson for technical assistance and Paige Hackney for typing the manuscript. We also thank Deborah Pinney, Mickey Williams, and Li-Fang Liang for helpful discussions.

This research was supported by Public Health Service grant 5R01 AI11722-10 from the National Institute of Allergy and Infectious Diseases. T.L.G. was supported by Public Health Service training grant 5T32-CA09109 from the National Cancer Institute.

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