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ATP Dependence of Vesicular Stomatitis Virus Transcription Initiation and Modulation by Mutation in the Nucleocapsid Protein

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We recently reported that vesicular stomatitis virus pol R mutants contain a template-associated N-protein alteration which allows for efficient readthrough of leader RNA termination sites in vitro (Perrault et al., Cell 35:175–185, 1983). We show here that in vitro RNA synthesis mediated by pol R virions is much more resistant to replacement of ATP by the analog β,γ-imido ATP than by wild-type virus (~50% inhibition versus ~95%). Characterization of β,γ-imido ATP-resistant and control products by size, polyadenylic acid content, frequency of initiation at the 3′ end of the template, and readthrough of the leader-*N* gene junction leads us to conclude the following: (i) most likely, the ATP dependence of the transcription process primarily reflects a requirement for initiation or entry of the polymerase at the 3′ end of the template; (ii) this requirement is largely bypassed in the mutant pol R viruses; (iii) the synthesis of small, internally initiated transcripts by wild-type virus is less dependent on ATP than that of leader RNA; and (iv) termination at leader RNA sites is not directly affected when β,γ-imido ATP is added before initiation of synthesis. These results are discussed in terms of the possible roles of ATP and the nucleocapsid protein in initiation and termination of vesicular stomatitis virus RNA synthesis.

Considerable efforts to understand the control of vesicular stomatitis virus (VSV) transcription have been expended in the last few years (for review see references 1 and 8). It is now thought that the virion polymerase (L protein), acting in concert with the NS phosphoprotein which appears to be necessary for directing the polymerase to its initiation site (13), normally enters the ribonucleoprotein (RNP) template at its very 3′ end and first synthesizes a plus-strand leader RNA 47 to 48 bases long (8). The polymerase complex then presumably reinitiates synthesis at the beginning of each of the five VSV structural genes (3′-N-NS-M-G-L-5′) without prior dissociation but with decreasing probability as it moves down the template. This stop-start transcription process is thus polar and attenuated. Pausing between gene junctions is presumably accompanied by a "chattering" process on a stretch of seven uridylate residues to give rise to mRNA polyadenylic acid [poly(A)] tails (12). Modification of mRNA 5′ ends by capping and methylation probably takes place on very short nascent chains (22, 25). Which of the three viral proteins is or are involved in the RNA modification reactions is not yet clear.

Although the depiction of the transcription process outlined above may well account for the behavior of the VSV polymerase when it gives rise to full-size mRNAs, several studies have shown that it does not always begin synthesis at the 3′ end of the template nor terminate at the ends of the viral genes. For example, frequent initiation at the beginning of some structural genes can occur in vitro without prior entry at the 3′ end, but these are apparently followed by abortive termination (14, 22, 28). Some of these events probably correspond to reiterative initiations by a "cycling polymerase" which may be a general feature of both procaryotic and eucaryotic transcriptases (31). More importantly for VSV, however, studies with *ts* mutants indicate that the same RNP template-polymerase protein complex is probably involved in the replication process (for review see reference 24). The requisite antitermination activity for synthesis of the full-size (~11 kilobases) plus-strand intermediate in genome RNA synthesis is normally found only in infected cells and requires protein synthesis (30). Some evidence suggests that newly synthesized N protein alone may be sufficient to cause readthrough by binding to nascent leader RNA chains and initiating assembly of progeny RNP (2, 15). However, significant readthrough activity can also occur in vitro in the absence of new N protein if base analogs are present (6, 7, 29), or under normal transcription conditions in the case of the VSV pol R mutants we have recently described (18). These studies suggest that the switch from transcription to replication may also depend on modification of one or more of the core viral proteins.

The effect of replacing ATP with analogs having nonhydrolyzable β,γ bonds during in vitro RNA synthesis has revealed a specific requirement for this ATP bond in the transcription process of VSV (27), as well as that of vaccinia virus (9) and eucaryotic RNA polymerase II (4). In the case of VSV, the effect of β,γ-imido ATP (AMP-PNP) is complex: synthesis is inhibited if the analog is added before initiation, but it leads to readthrough synthesis if added after a preinitiation step in the presence of ATP and CTP (29). Since we recently showed that VSV pol R mutants read through the leader RNA termination sites efficiently in vitro under normal transcription conditions, as a result of an N-protein alteration (18), we reasoned that these mutants might also show an altered dependence on ATP. The results presented here show that this is indeed the case, but that, somewhat unexpectedly, readthrough synthesis of the leader-*N* gene junction in these mutants is not differentially affected by addition of AMP-PNP before initiation. Furthermore, we present evidence that internal initiation by the wild-type VSV polymerase is less dependent on hydrolysis of the β,γ bond of ATP than is initiation at the 3′ end of the template.

MATERIALS AND METHODS

Growth, source, and purification of virus. Sources of wild-type standard VSV Indiana (Mudd-Summers strain) and HR

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strain (Toronto), as well as of defective interfering (DI) particles from wild-type (DI *0.22* or WT-T) or pol R1 VSV (DI *0.21*), were described previously (18, 19). Details of the heat selection procedure which gave rise to pol R1 and pol R2 VSV mutants will be described elsewhere. Virus purification from infected BHK-21 cells was carried out on sucrose and tartrate gradients as before (21).

In vitro polymerase assay of standard virus and DI particles. All polymerase reactions were carried out in 250-μl volumes and, unless otherwise stated, contained the following: 50 mM Tris-acetate; 8 mM magnesium acetate; 0.3 M potassium acetate; 1 mM each ATP, GTP, and CTP; 0.1 mM UTP (all nucleotide triphosphates from Calbiochem); 2 mM dithiothreitol; 0.1% Nonidet P-40; 200 μg of virus per ml; and 50 or 100 μCi of [^{3}H]UTP (ICN; 38 Ci/mmol) per ml, for standard and DI particle-containing reactions, respectively. Under these conditions, the specific activities of product RNAs were equivalent to 215 and 430 kcpm/μg. Reaction mixes of different pH values were obtained by using appropriate concentrated stock solutions of Tris-acetate buffer. The pH values of reaction mixes refer to measurements taken after addition of all components except radioactive precursor and virus. Where indicated, ATP was replaced by 1 mM AMP-PNP (Boehringer Mannheim, lot 742153). Since AMP-PNP preparations can contain variable amounts of contaminating ATP which can increase on storage (16), we have determined the amount of ATP in our preparations by the sensitive method of Gonzalez and Garcia-Sancho (10). The ATP content was 0.31% after 21 months of storage at −20°C, and all experiments were carried out before this time, using this same lot number of preparation. Assay of labeled precursor incorporation and purification of products free of their RNP templates by CsCl centrifugation were described previously (18). In experiments in which products were not freed of their templates but examined directly on agarose gels, the transcripts were separated from labeled unincorporated precursors by centrifugation through a G-50 Sephadex column as described before (18). This step effectively removes all oligonucleotide chains less than 20 bases long.

Assay of readthrough transcripts and digestion of poly(A) tracts. End-labeling of VSV genome RNA with cytidine 3′,5′-bis-[^{32}P]phosphate and RNA ligase, annealing of products to labeled probe, digestion with RNases A and T_1, and analysis of resistant duplexes on 15% polyacrylamide gels were all described previously (18).

To remove poly(A) tails from transcripts, purified product RNAs were spun from ethanol suspensions, combined with sufficient carrier RNA (yeast total soluble RNA) to make a total of ~25 μg, and suspended in ~15 μl of 10 mM Tris chloride (pH 7.7)–1 mM EDTA containing ~15 μg of oligodeoxythymidylic acid per ml. The sample was then heated at 100°C for 10 s and then cooled to room temperature, and an equal volume of 2× RNase H buffer (0.1 M Tris chloride, pH 7.7, 20 mM $MgCl_2$, 2 mM EDTA, 20 mM dithiothreitol, 20% glycerol) was added, followed by incubation at 50°C for 1 min. Digestion was then carried out by adding 0.5 U of *E. coli* RNase H (Enzo Co.) in 5 μl and incubated at 30°C for 45 min. The digested products were then repurified by proteinase K treatment and phenol-chloroform extraction as before (18).

Agarose gel analysis. Agarose gel (1%) analysis of glyoxal-denatured RNA samples and fluorography were carried out as described previously (18). Quantitation of bands was obtained by densitometry and corrected for nonlinearity by using standard curves derived from graded film exposures.

RESULTS

Inhibition of in vitro polymerase activity from standard particles of wild-type and pol R VSV mutants in the presence of AMP-PNP. In view of previous reports on the effects of AMP-PNP on VSV endogenous polymerase activity and readthrough synthesis (see above), we tested the effect of this analog added in place of ATP in in vitro reactions catalyzed by standard pol R mutant virions. Figure 1A shows the kinetics of incorporation of [^{3}H]UMP into wild-type VSV transcripts in the presence of ATP or AMP-PNP. The results obtained confirm those of Testa and Banerjee (27) and indicate almost complete inhibition of activity (~97%) in the presence of the analog. In contrast to wild-type virus, both mutant viruses showed substantial incorporation in the presence of AMP-PNP, representing 55 and 48% of the ATP activity after 3 h of reaction for pol R1 and pol R2, respectively (Fig. 1B and C). Note also that the activities from the mutant viruses under standard ATP conditions were roughly 50% less than wild-type virus as reported earlier (20). Specific activities of different virus preparations varied somewhat, especially with time of storage before assay, but the mutant pol R viruses always showed a comparable major difference in AMP-PNP-resistant activity relative to wild-type virus.

We have also observed the same phenomenon when transcribing virus cores instead of whole virus, indicating that the AMP-PNP-resistant activity of the pol R mutant does not depend on envelope components (not shown). A different source of "wild-type" virus, the HR VSV Toronto strain, which was obtained by a heat selection protocol similar to that employed for generating our pol R mutants, showed results identical to those found with the wild-type Mudd-Summers strain, indicating that the heat selection procedure per se does not necessarily lead to AMP-PNP resistance. Since both mutants were isolated independently and probably contain few mutations compared to the wild type (18), these results suggest that the ATP dependence of the VSV transcription process is specifically altered by the N-protein mutations in pol R viruses.

Differential effects of pH and salt concentration on ATP and AMP-PNP activities of wild-type and pol R standard VSV. Talib and Hearst have recently reported that internal initiation at the beginning of the VSV N gene in vitro is resistant

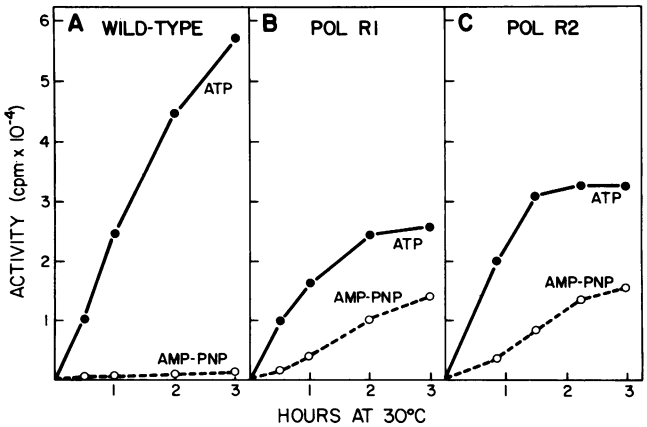

FIG. 1. Incorporation of [^{3}H]UMP into standard wild-type (Mudd-Summers) VSV RNA transcripts in vitro in the presence of ATP or AMP-PNP. The pH of the reaction mixes was 7.8, and the activity shown refers to 10-μl samples (see the text).

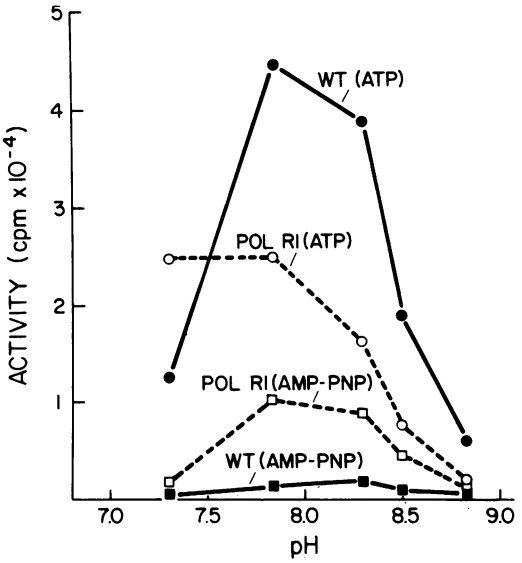

FIG. 2. In vitro activity of standard wild-type (Mudd-Summers) and pol R1 VSV in the presence of ATP or AMP-PNP as a function of pH. The activities plotted refer to incorporation in 10-μl samples after 2 h of reaction time.

to aurintricarboxylic acid and vanadyl ribonucleoside complexes (26). On the basis of these results they have suggested the existence of two types of polymerase activities in VSV, one for the synthesis of leader and one for mRNA. Since the activity displayed by pol R VSV mutants in the presence of AMP-PNP also raised the possibility of a second polymerase, we tested whether different reaction conditions might be required for optimizing this synthesis. The effects of various pH conditions are shown in Fig. 2. Wild-type VSV under standard ATP conditions showed maximal incorporation around pH 7.8 with decreasing values on both acidic and basic sides of this optimum. In contrast, pol R1 VSV showed comparable activity at pH 7.3 versus pH 7.8 in the presence of ATP. These results were reproducibly obtained with different batches of virus and also with pol R2 virus (data not shown). This enhanced activity at low pH for pol R virus may be related to the charge difference in the N protein of the RNP template common to both mutants (18).

The AMP-PNP-resistant activities of both wild-type and pol R1 virus displayed a small but significantly more basic pH optimum than their ATP activities (Fig. 2). Note, for example, that the percentage of AMP-PNP-resistant activity in relation to ATP activity for pol R1 virus increased from ~12% at pH 7.3 to ~63% at pH 8.5. For wild-type virus, the AMP-PNP-resistant activity remained very low but was nevertheless higher at pH 8.3 than at pH 7.8, in contrast to its ATP activity. These slight differences could possibly reflect a difference in the binding of the AMP-PNP substrate to the active site on the polymerase enzyme. In any case, no major difference in pH optimum could distinguish between the ATP control and the AMP-PNP-resistant activities.

The requirement for relatively high monovalent cation salt concentration in VSV polymerase reactions is thought to reflect the need for removal of the membrane M protein inhibitor bound to the RNP template (23). The effect of potassium acetate concentration on the ATP control and AMP-PNP-resistant activities of wild-type and pol R1 virus is displayed in Fig. 3. As shown previously by Breindl and Holland (3), wild-type virus required 0.2 to 0.3 M potassium acetate for optimum activity in the presence of ATP (Fig. 3A). In contrast, pol R1 virus required 0.3 to 0.4 M potassium acetate for maximum incorporation (Fig. 3B). As in the case of pH optimum differences between wild-type and pol R VSV, this salt optimum difference most likely reflects the charge difference in the N protein of the pol R RNP templates, although pol R1 virus also contains a mutation in its M protein which could play a role in this change (18). The optimal salt concentration for the AMP-PNP-resistant activity of pol R1 virus was slightly lower (~0.30 M versus ~0.35 M) than the ATP control activity (Fig. 3B). Little or no difference was seen between the wild-type ATP control and AMP-PNP-resistant activities (Fig. 3A). These results suggest that the AMP-PNP-resistant activity of both wild-type and mutant viruses most likely requires the removal of M protein from the RNP template as in the case of the ATP activity.

Since we found similar pH, salt, and divalent cation requirements (the latter not shown) for both ATP control and AMP-PNP-resistant incorporations, we conclude that both of these activities are probably carried out by the same VSV polymerase.

Characterization of AMP-PNP-resistant transcripts from wild-type and pol R standard virus particles. We have previously reported that transcribing cores of pol R1 virus, for the most part, gave rise to the same species of mRNA as wild-type virus under standard ATP conditions, except for longer and more heterogeneous poly(A) tails (20). The agarose gel analysis of glyoxal-denatured products (Fig. 4A, lanes b to e) indicated that this was also true for whole virus instead of cores. Note that approximately equal amounts of labeled products were loaded in each of these lanes. The differences between lanes b versus c (wild type) and lanes d versus e (pol R1) illustrate the dramatic sharpening of transcript bands after removal of the heterogeneously sized poly(A) tails. The latter were digested by first annealing the transcripts to oligodeoxythymidylic acid and treating with RNase H, which specifically degrades the RNA strands of RNA-DNA duplexes. Aside from bands corresponding to G, (1,665 nucleotides [nt]), N (1,326 nt), and NS + M (815 and 831 nt) mRNAs, both wild-type and mutant viruses variably synthesized two higher-molecular-weight species (A and B) which most likely represent dicistronic mRNAs (N + NS and M + G) lacking intervening poly(A) tails (manuscript in preparation). Note also that pol R1 virus synthesized far more heterogeneously sized transcripts migrating between the 5S

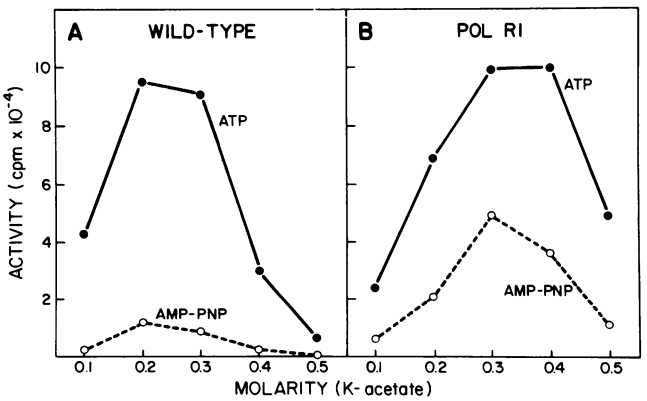

FIG. 3. In vitro polymerase activity of standard wild-type (HR) and pol R1 VSV in the presence of ATP or AMP-PNP as a function of salt concentration. The activities were measured in pH 8.3 reactions and refer to incorporation in 25-μl samples after 3 h of reaction time.

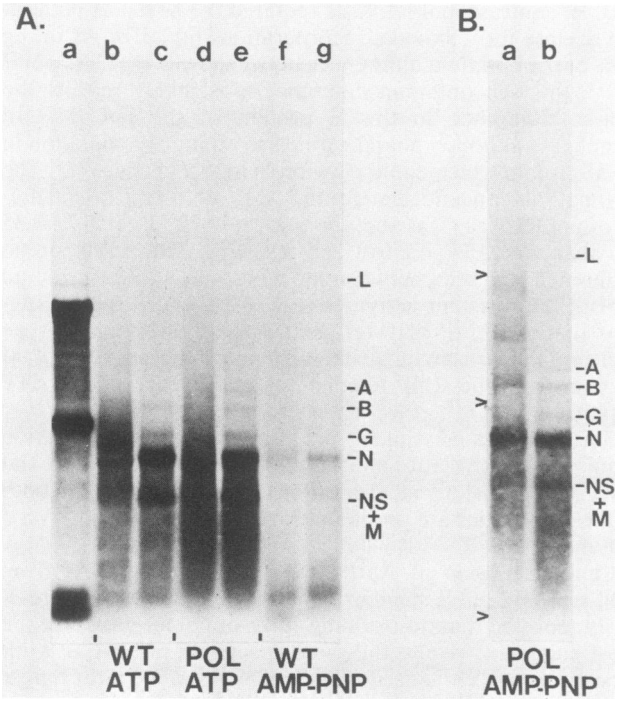

FIG. 4. Agarose gel analysis of [^{3}H]UMP-labeled transcripts from standard wild-type (HR strain) and pol R1 VSV after denaturation with glyoxal. Gels were processed for fluorography as described in the text. (A) Lane a, [^{3}H]uridine-labeled HeLa cell rRNA and tRNA; lanes b and d, wild-type and pol R1 VSV transcripts made in the presence of ATP; lanes c and e, same as b and d but after removal of poly(A) tails; lanes f and g, wild-type VSV transcripts made in the presence of AMP-PNP before and after removal of poly(A) tails. Approximately one-fourth as many counts per minute were loaded in these last two lanes. All products shown were synthesized at pH 8.5, but similar results were obtained at pH 7.8. (B) Lanes a and b, pol R1 VSV transcripts made in the presence of AMP-PNP at pH 7.8, before and after removal of poly(A) tails. Arrowheads indicate position of marker HeLa cell RNAs run on a parallel lane.

RNA marker and the NS + M mRNA band than did wild-type virus (Fig. 4A, lanes d and e). These correspond mostly to readthrough transcripts containing leader RNA linked to various lengths of N mRNA, as documented earlier (18, 20). Both pol R1 and wild-type products were found to be similar whether synthesized at pH 8.5 (Fig. 4A) or pH 7.8 (not shown). However, at pH 7.3 only small-size products (<400 nt) were produced by both types of viruses (not shown).

Figure 4A (lanes f and g) shows the results of a similar analysis of wild-type virus transcripts made in the presence of AMP-PNP. Approximately one fourth as many counts per minute of products were loaded in these lanes. In contrast to transcripts made in the presence of ATP, wild-type virus gave rise to a high proportion (41%) of small-size RNAs (<200 nt), as well as larger products (Fig. 4A, lane f). These results superficially resemble those reported by Chinchar et al. (7), who have analyzed AMP-PNP-resistant products from wild-type virus after a preinitiation step in the presence of ATP and CTP. After removal of poly(A) tails (Fig. 4A, lane g), relatively little change was observed in the pattern, except for a slight sharpening of the major high-molecular-weight product and the appearance of trace amounts of sharp bands corresponding to NS + M and G mRNAs (the latter were more clearly seen on longer exposures of the fluorograph). The major large RNA band, either before or after RNase H digestion, migrated at or near the position expected for N mRNA lacking significant lengths of poly(A) tails. Note that a similar species was also synthesized by both wild-type and pol R1 virus in the presence of ATP (Fig. 4A, lanes b and d; 20). On the whole, therefore, AMP-PNP prevents the synthesis of almost all full-size wild-type VSV transcripts containing poly(A) tails. A more quantitative analysis of this inhibition is presented in Table 1, where the fraction of the total RNA represented by individual major bands corresponding to N, NS + M, and small RNAs (<200 nt) was determined from densitometer tracings of the fluorograph (see Materials and Methods).

The nature of the small, wild-type virus products which are relatively resistant to AMP-PNP inhibition is intriguing since their relative abundance (≥90% of all products on a molar basis, assuming that their average size is no greater than 100 nt and that that of all other transcripts not represented in the major bands is no larger than that of G mRNA) implies that the VSV polymerase can still efficiently initiate synthesis but, for the most part, fails to elongate these chains beyond 200 nt or so. As will be described below, the majority of these small products do not correspond to initiations at the 3′ end of the template.

The more abundant AMP-PNP-resistant transcripts synthesized by the mutant pol R1 virus are shown in Fig. 4B. In contrast to wild-type virus, most or all products were large, with a major species again migrating near or at the position of N mRNA lacking poly(A) tails. Small amounts of higher-molecular-weight species, including trace amounts of genome-size RNA, were also clearly synthesized. Possibly, as in the case of wild-type virus, the N-mRNA-size species is the same as that synthesized in the presence of ATP (see above) and is more resistant to AMP-PNP inhibition than poly(A)-containing products. Removal of poly(A) tails from the pol R1 AMP-PNP-resistant transcripts again generated small amounts of sharp bands corresponding to NS + M, G, A, and B, as well as additional N mRNA species (Fig. 4B, lane b). Disappearance of the largest RNAs after RNase H treatment could conceivably indicate the presence of transcripts containing intervening poly(A) linking mRNA species. The latter have been reported to be synthesized in small amounts (3 to 5%) by wild-type virus in vitro (11). However, these transcripts could also represent readthrough replicationlike products without intervening poly(A) which, because of their large size, would be very susceptible to trace amounts of single-stranded RNA-specific RNases in the

TABLE 1. Inhibition of VSV RNA synthesis in the presence of AMP-PNP[a]

Virus	Measured fraction	% of ATP products	% of AMP-PNP products	% Inhibition
Wild-type virus	Total			90
	N	26	14	95
	NS + M	21	10	95
	<200 nt	9.2	41	55
Pol R1 virus	Total			60
	N	18	19	58
	NS + M	24	21	65
	<200 nt	5.5	8.0	42

[a] The lanes corresponding to the RNase H-treated samples in the fluorographs shown in Fig. 4 were scanned with a densitometer, and the various fractions were quantitated as described in the text. The percent inhibition of individual fractions was calculated from their relative abundance in a given reaction and the overall total percent inhibition of incorporation.

RNase H preparation. This is more compatible with the observed increase in heterogeneously sized molecules below the NS + M mRNA band. Whether any of these pol R1, AMP-PNP-resistant transcripts span the leader-*N* gene junction is more fully explored in the next section. Table 1 summarizes the observed inhibition of individual pol R1 transcript fractions. We conclude from these experiments that AMP-PNP-resistant RNA synthesis by pol R1 virus represents, at least in part, the synthesis of mRNA species similar in size to those seen in the presence of ATP. The transcription process in this mutant virus therefore appears to be less dependent on ATP than that of wild-type virus. Qualitatively similar results were obtained with pol R2 virus (not shown).

Readthrough of plus-strand leader gene termination site in the presence of AMP-PNP. We have shown previously that once the VSV polymerase initiates synthesis at the 3′ end of the wild-type virus RNP template, it reads through the leader-*N* gene junction (residues 48 to 50 from the 3′ end) with ~10% frequency under normal in vitro transcription conditions, although it rarely if ever terminates past the *N* gene. In contrast, the same wild-type virus polymerase reads through this junction with ~80 to 85% frequency when reconstituted with pol R virus RNP templates, and again terminates mostly within the *N* gene (18). Since others have reported that genome-size readthrough products (29), or leader-*N* gene linked transcripts (7), are also synthesized in the presence of AMP-PNP upon a preinitiation step in the presence of ATP and CTP, we reasoned that such readthrough synthesis might be in part responsible for the activity of the pol R mutants when AMP-PNP was added before initiation as in our experiments described above. We therefore tested this hypothesis by using the assay we have previously developed for detecting readthrough, namely, protection of a 3′-end-labeled genome probe from nuclease digestion after annealing of product RNAs (18). This assay also quantitates the relative frequency of initiation at the 3′ end of the template, assuming all such initiations yield molecules long enough to give rise to stable hybrids. We have shown previously that 3′-end transcripts 21 bases long can be recovered quantitatively in this assay (21).

We carried out a series of six parallel polymerase reactions containing equal amounts of standard wild-type, pol R1, or pol R2 virus in the presence of ATP or AMP-PNP as for Fig. 1. Incorporation of [^{3}H]UMP in the presence of AMP-PNP versus ATP was in this case 9.6% for wild type, 46% for pol R1, and 36% for pol R2 after 2 h of reaction. The products were purified free of their RNP templates and annealed in three sets of 10-fold increasing concentrations to a constant amount of 3′-end-labeled genome probe. RNase-resistant duplexes were then analyzed on a 15% polyacrylamide gel (Fig. 5). The total amount of ^{32}P label protected in the duplexes reflects the relative frequency of 3′-end initiation events among the various reaction products when the probe is in excess (the first two lanes of each set). Note that the labeled genome probe consisted of relatively small fragments generated by contaminating nucleases in the ligase reaction employed for its preparation (Fig. 5B, lane g). However, all of these fragments were larger than leader RNA size (47 nt). The appearance of larger duplexes after annealing is thus indicative of readthrough of the leader-*N* gene junction, but does not necessarily reflect the size of these readthrough molecules.

As shown previously (18), wild-type VSV synthesized very few readthrough transcripts in the presence of ATP and almost all of the label was found in leader-size duplex

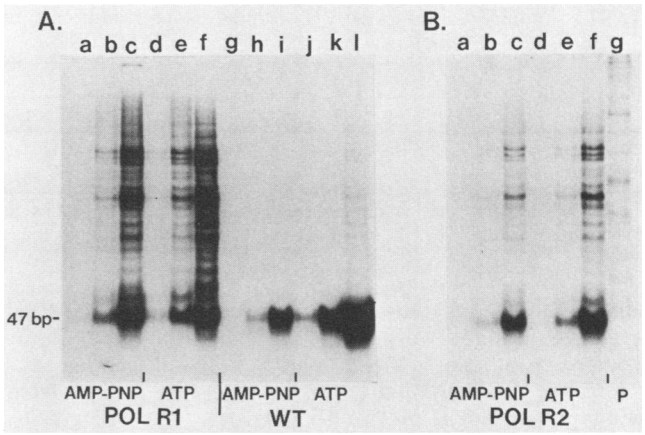

FIG. 5. Assay for readthrough of the leader-*N* gene junction in wild-type (Mudd-Summers) and pol R standard VSV transcripts in the presence of ATP or AMP-PNP. RNase-resistant duplexes were generated by annealing three different concentrations of product RNAs (0.016, 0.16, and 1.6 μg) to ~1 μg of 3′-end-labeled genome RNA and electrophoresing on a nondenaturing 15% polyacrylamide gel (see the text). (A) Lanes a, b, and c, increasing concentrations of AMP-PNP-resistant pol R1 products; lanes d, e, and f, pol R1 ATP control products; lanes g, h, and i, wild-type AMP-PNP-resistant products; lanes j, k, and l, wild-type ATP control products. (B) Lanes a, b, and c, pol R2 AMP-PNP-resistant products; lanes d, e, and f, pol R2 ATP control products; lane g, labeled genome probe without nuclease digestion. No bands were seen after digestion of this probe annealed in the absence of products (not shown).

molecules (Fig. 5A, lanes j, k, and l). In the presence of AMP-PNP, no increase in readthrough transcripts was observed, and densitomer tracings of the relevant lanes (Fig. 5A, lanes g and h versus j and k) showed that leader RNA synthesis was inhibited nearly as much (~80%) as overall synthesis of RNA (Table 2). Both pol R1 and pol R2 viruses, as shown before (18), gave rise to a high proportion (~76%) of readthrough transcripts in the presence of ATP as reflected by the presence of larger-size duplexes in addition to leader RNA (Fig. 5A and B, lanes d, e, and f). In the presence of AMP-PNP, the synthesis of both readthrough transcripts and leader RNA was inhibited, again roughly to the same extent (~49 and ~66%, respectively) as overall RNA synthesis (Fig. 5A and B, lanes a, b, and c; Table 2). Note that no significant amounts of hybrids smaller than leader RNA were observed under all conditions even though transcripts >20 bases long would have been detected readily (21). We thus conclude that, under our conditions of transcription with AMP-PNP added before initiation of transcription, the analog has no major differential effect on readthrough of the leader-*N* gene junction for either wild-type or pol R virus. Furthermore, unless AMP-PNP forced the VSV polymerase to make very short chains (<20 bases long), initiation at the 3′ end of the VSV template, whether it was subsequently followed by termination at the leader RNA site or not, was nearly as sensitive to AMP-PNP inhibition as overall incorporation of [^{3}H]UMP into RNA.

On the basis that small products synthesized by wild-type virus were only 55% inhibited by AMP-PNP (Table 1) whereas leader RNA was 80% inhibited (Table 2), we also conclude that a major fraction of these small RNAs must be initiated internally on the VSV template. Such internal initiations are either less dependent on the β,γ bond of ATP than initiations at the 3′ end of the template, or more likely totally independent of this requirement if leader RNA under

TABLE 2. Inhibition of leader RNA synthesis and readthrough of the leader-*N* gene junction in the presence of AMP-PNP[a]

Virus source	% Inhibition		
	Total RNA	Leader synthesis	Readthrough
Wild type	90	80	ND
Pol R1	54	49	66

[a] The appropriate lanes of the autoradiograph shown in Fig. 5A were quantitated as before (18). ND, Not determined.

our conditions of transcription represents ~6% of total products (or about two-thirds of our small RNA fraction) as reported by others (5, 14).

Effect of AMP-PNP on in vitro polymerase activity from wild-type and pol R mutant DI particles. The endogenous polymerase in DI particles derived from wild-type virus, for the most part, synthesizes only a 46-nt-long minus-strand leader RNA in vitro (for review, see reference 17). In contrast, the polymerase in pol R mutant-derived DI particles reads through the minus-strand leader termination site and gives rise to heterogeneously sized large RNA products (19). Since reinitiation of chain synthesis does not occur internally on DI templates, in contrast to standard virus templates, we tested the effects of AMP-PNP in this simpler transcription process. Wild-type DI particle-mediated synthesis was not quite as sensitive to AMP-PNP inhibition as standard wild-type virus at pH 7.8 (85 versus 97% inhibition) (Fig. 6A). The effect of pH and salt concentration on both ATP control and AMP-PNP-resistant synthesis of wild-type DI particles (not shown) was similar to that seen for standard virus except for a significantly higher AMP-PNP-resistant activity for wild-type DI particles at higher pH (~26 and ~40% of ATP activity at pH 8.3 and 8.5, respectively). Polymerase activity in pol R1 DI particles was, on the other hand, more resistant to AMP-PNP inhibition than that in wild-type DI particles at pH 7.8 (69 versus 85% inhibition), but this difference was somewhat less than that seen for standard virus particles (see Fig. 1). Again, the effect of pH and salt concentration on pol R1 DI activities was similar to that seen for standard particles except for reduced activity at pH 7.3 (not shown). Note that [^{3}H]UMP incorporation in pol R1 DI particles was much higher than in wild-type DI (see scale difference between Fig. 6A and 6B) because of the large increase in size of the products as reported previously (18, 19). Agarose gel analysis of both wild-type and pol R1 DI particle products, as described above for standard particles, resulted in profiles very similar to those found in our previous studies (19) and showed that AMP-PNP-resistant transcripts were qualitatively the same as those synthesized in the presence of ATP (not shown). We therefore conclude from these experiments that, although wild-type DI polymerase activity is more resistant to AMP-PNP inhibition than wild-type standard virus, especially at high pH, initiation of synthesis at the 3′ end of DI templates from pol R virus is also significantly more resistant than that of wild-type virus DI particles. These results further support our hypothesis that the main effect of AMP-PNP, when added at the start of VSV polymerase reactions, is inhibition of RNA synthesis initiated at the 3′ end of the template.

DISCUSSION

The main conclusions of this paper are that in vitro transcription by VSV pol R mutants shows less dependence on cleavage of the β,γ bond of ATP than transcription by wild-type virus and that this ATP requirement primarily reflects some initiation or preinitiation events required for synthesis beginning at the 3′ end of the template. Synthesis of the most abundant VSV mRNAs (N, NS, and M) was found to be much more resistant to replacement of ATP by AMP-PNP in mutant pol R viruses than in wild-type virus (~62 versus 95% inhibition). Leader RNA and readthrough products spanning the leader-*N* gene junction also behaved similarly. Therefore, when AMP-PNP replaces ATP before initiation of RNA synthesis, readthrough synthesis of leader termination sites is not directly affected. Since we detected a small fraction of very large RNA products from pol R mutants under these conditions, AMP-PNP could also lead to enhanced readthrough of gene junctions beyond the leader termination site.

Curiously, wild-type virus also synthesized relatively large amounts of small internally initiated transcripts in the presence of AMP-PNP. Since these were <200 nt long and lacked poly(A) tails, they probably correspond to the 5′ ends of one or more VSV mRNAs. Wild-type VSV polymerase has been known for some time to give rise to such internally initiated, abortive transcripts under normal in vitro transcription conditions (see Introduction). In any case, our results suggest that it is not initiation of RNA chains per se, i.e., formation of the first phosphodiester bond, which requires hydrolysis of the β,γ bond of ATP, but some other event specific to the start of RNA synthesis at the 3′ end of the template.

One important consideration regarding our conclusions focuses on the possibility that contaminating levels of ATP in the AMP-PNP preparation (≤3 μM ATP for 1 mM AMP-PNP) might be responsible for the effects observed. It must first be pointed out that the experiments of Testa and Banerjee (27) have clearly shown that the requirement for high concentrations of ATP in VSV RNA synthesis can be bypassed by first preinitiating chains in the presence of 1 mM ATP and 0.5 mM CTP. This requirement could not be met by equivalent concentrations of AMP-PNP, whereas elongation could take place in the presence of low concentrations of ATP (K_m = 25 μM) or in the presence of 1 mM AMP-PNP (27). Although it is conceivable that, in the AMP-PNP experiments reported here, RNA chains were actually initiated with the very low levels of contaminating ATP, this would be in direct conflict with the results of Testa and Banerjee described above. We therefore conclude that our observations are best explained by a requirement for cleav-

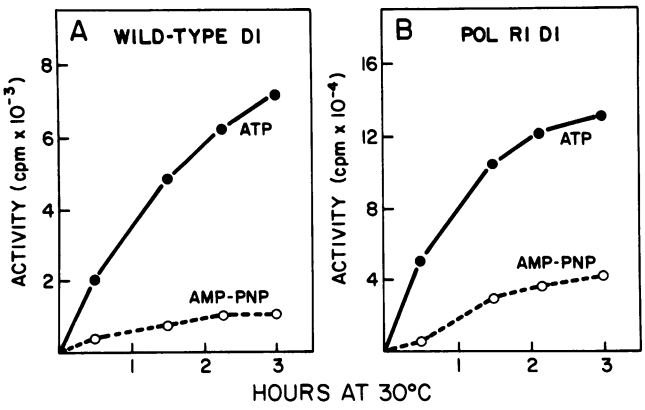

FIG. 6. Incorporation of [^{3}H]UMP into wild-type (Mudd-Summers) and pol R1 VSV DI particles in the presence of ATP or AMP-PNP. Activities plotted refer to incorporation in 10-μl samples from reactions carried out at pH 7.8.

age of the β,γ bond of ATP for leader RNA synthesis, which precedes mRNA synthesis, and that such requirement is either unnecessary or less stringent in the case of abortive, internally initiated transcripts.

A second important consideration reflects the possibility that AMP-PNP does not inhibit initiation of RNA chains at the 3′ end of the template but instead causes very early termination, yielding products <20 bases long. Some or all of these could have remained undetected in our assay, which relies on the formation of stable duplex molecules with a 3′-end-labeled genome probe. Although we cannot rule out this possibility, it seems unlikely to us because no duplexes smaller than leader RNA size (48 base pairs) were observed under all conditions examined (Fig. 5). Furthermore, no discernible effect of AMP-PNP on termination could be seen for any of the pol R transcripts which extended past the leader RNA termination site (Fig. 5A and 5B, lanes a to f).

What allows pol R mutants to synthesize RNA relatively efficiently in the presence of AMP-PNP is not yet clear. One seeming explanation might be that the mutant virus has a higher affinity (lower K_m value) for ATP than wild-type virus and is therefore able to utilize more efficiently the small amounts of ATP present in the AMP-PNP-containing reaction. Indeed, we have measured the K_m values for ATP and AMP-PNP utilization by pol R1 standard virus, and these were found to be both the same and equal to ~125 μM, which is significantly lower than the K_m value of ~500 μM reported for ATP utilization during initiation of wild-type VSV synthesis (27). A more detailed kinetic analysis of ATP dependence of VSV wild-type and pol R mutant transcription will be reported elsewhere (J. Perrault, manuscript in preparation). Despite the apparent lower K_m for ATP displayed by the pol R viruses, it seems highly unlikely that this is sufficient in itself to explain the ~50% remaining activity in the presence of AMP-PNP, since this represents at most an ATP concentration of 3 μM which is far below the K_m value. As in the case of wild-type virus, it again seems far more likely that the mutant virus can utilize AMP-PNP for initiating RNA chains but, in this case, more efficiently than can wild-type virus.

If, as indicated above, mutant pol R viruses and, to a small extent, wild-type virus can bypass the requirement for a hydrolyzable β,γ bond of ATP, what then could this cleavage be used for? It seems likely to us that the change in ATP dependence in the pol R mutants is caused by the same template N-protein mutation that is responsible for their enhanced readthrough synthesis of the leader-*N* gene junction under normal transcription conditions. One possibility is that ATP is involved in some sort of crucial modification of the template N protein, of one of the other viral proteins interacting with the RNP template, perhaps via protein phosphorylation, or of both. Alternatively, ATP hydrolysis could be coupled to some energy-requiring process necessary for correct positioning of the polymerase complex at the 3′ end. On the basis of our previous results with pol R mutants, we have already speculated that the ability to read through leader gene termination sites may be controlled by a modification of at least some of the N-protein subunits on the RNP template (18). Whether this modification exists and might be involved in both phenomena remains to be seen. It is also tempting to speculate that the same N mutation(s) affecting the putative modification is responsible for the aberrantly long and heterogeneous poly(A) tails found on pol R mutant mRNA transcripts (20). Perhaps related to this is our observation that the major large RNA species found in AMP-PNP-resistant products from both wild-type and pol R standard viruses comigrated with N mRNA lacking appreciable poly(A) tails (Fig. 4). A similar species is also made in the presence of ATP, where it represents only a small fraction compared to polyadenylated N mRNA. Studies to be reported elsewhere show that this species can be methylated to the same extent as other viral mRNAs in the presence of *S*-adenosylmethionine. This raises the possibility that synthesis of this putative N mRNA lacking poly(A) is somewhat more resistant to AMP-PNP inhibition than that of its polyadenylated counterpart.

It is difficult at present to prove the hypothesis that the same N-protein mutation is involved in all of the above phenomena. Pol R VSV could contain more than one relevant mutation, and we have not yet found conditions for selecting wild-type revertants. However, insofar as the two mutants, pol R1 and pol R2, were independently isolated and appear to contain very few base changes compared to wild type, and all of the above-mentioned phenotypic traits covary (18), this hypothesis seems well worth pursuing.

It is interesting that, although RNA synthesis by the mutant pol R DI particles is more resistant to AMP-PNP inhibition than that by wild-type virus DI particles, the latter is nevertheless significantly more resistant than that of the parent standard virus particles, especially when the reactions are carried out at higher pH. This difference in ATP requirement could perhaps reflect distinctions in the modification of standard versus DI RNPs and be related to the competitive advantage enjoyed by the latter during in vivo replication (17).

Further work to elucidate the relationship between the pol R VSV mutations, the ATP dependence of initiation of RNA synthesis at the 3′ end of the RNP template, and the crucial switch from transcription to replication accompanying readthrough of the leader gene termination sites should prove rewarding.

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