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Differential Effect of ATP Concentration on Synthesis of Vesicular Stomatitis Virus Leader RNAs and mRNAs

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Cleavage of the β-γ bond of ATP is required for wild-type (wt) vesicular stomatitis virus transcription in vitro. Recent findings have established that a domain-specific phosphorylation of the virus NS protein is necessary for activity. We report here that RNA synthesis catalyzed by purified standard wt virions responded cooperatively to various ATP concentrations, with half-maximal activity at ~500 μM. In contrast, mutant polR1 standard virions and wt defective interfering particles both showed conventional Michaelis-Menten kinetic profiles with K_m values of ~143 and ~133 μM, respectively. The former synthesize readthrough products of the leader-N gene junction in addition to plus-strand leader RNA and mRNAs, whereas the latter synthesize only minus-strand leader RNA. The cooperative response of wt virus products, however, was specific to mRNAs; the small fraction of the total products corresponding to plus-strand leader approximated Michaelis-Menten behavior. Since the unique phenotype of the polR mutants correlates with the synthesis of replicationlike products in vitro, the affected ATP-requiring function most likely regulates both transcription and replication. We suggest that this mutated function involves phosphorylation of viral proteins.

The prototype rhabdovirus, vesicular stomatitis virus (VSV), is an enveloped virus containing a negative-stranded RNA genome of 11,161 nucleotides. All five virus-coded proteins (3′-N-NS-M-G-L-5′) are found in the virion structure. Although transcription of monocistronic virus mRNAs by the virion-associated polymerase complex (L, NS, and N-RNA template) has been extensively studied for the last decade, several aspects of this process are not well understood (for a recent comprehensive review, see reference 3). Recent findings have clearly established that phosphorylation of a specific domain of the NS protein is required for transcription activity (10, 19). Which of the various facets of the transcription process are affected by this particular phosphorylation remains to be explored. In particular, it is not clear whether the high ATP concentration requirement for in vitro transcription (4, 47) and the obligatory cleavage of the β-γ bond of this nucleotide (20, 36, 47) reflect only the aforementioned phosphorylation of the NS protein or whether additional ATP-requiring events are involved. Answers to these questions may be pertinent to other viral and cellular transcription processes which also require the energy-rich bond of ATP (7, 15, 18, 28).

Replication of the VSV genome may also be controlled by protein phosphorylation, but much less is known about this process (3). The mandatory switch between transcription and replication is apparently coupled to simultaneous assembly of progeny RNA with the core N protein (1, 5, 30). Transcription involves termination and release of completed mRNAs at the gene junctions (6); replication depends on readthrough or antitermination to produce a full-size, plus-strand intermediate which then serves as the template for progeny minus strands (45). Whatever the mechanism may be for this critical switch, it should be emphasized that all templates, whether transcriptive or replicative, are not naked RNA molecules but ribonucleoprotein (RNP) structures containing ~1,500 copies of the N protein tightly associated with the RNA (17, 45, 50). Furthermore, the in vitro transcription process apparently begins with the synthesis of a 47-base plus-strand leader RNA complementary to the exact 3′ end of the template (13, 16); this is then followed by sequential initiation of downstream mRNAs via a processive, but attenuated, stop-start mechanism (24). The switch to replication thus takes place first at the leader-N gene junction and does not involve a separate entry site (2, 3).

Replication of minus-strand progeny RNA involves the synthesis of an analogous 46-base minus-strand leader RNA copied from the 3′ end of the full-size plus strand in vivo (26); antitermination at this site is presumably also dependent on concurrent assembly (5). The synthesis of this minus-strand leader is observed in vitro when VSV defective interfering (DI) particles are used as templates (42, 43); the truncated genome they contain has the same 3′-end sequence as the plus-strand genomic intermediate (25, 31).

Although newly synthesized N protein is undoubtedly required for synthesis of competent progeny RNP templates, some evidence suggests that a reaction(s) requiring ATP is also involved in the switch from transcription to replication. For example, transcription using purified wild-type (wt) virus is drastically inhibited if the analog β,γ-imido ATP (AMP-PNP) is substituted for ATP (20, 36, 47). This analog cannot be used as a substrate for protein kinase or in other reactions which cleave the β-γ bond of ATP. However, if initiation is first carried out in the presence of ATP and CTP, subsequent elongation can take place in the presence of the analog and leads to synthesis of full-size, plus-strand RNA in the absence of any added protein (49). Other in vitro transcription studies using nucleotide analogs have also shown synthesis of partial readthrough transcripts (9, 11, 22). Moreover, we have reported that purified VSV polR mutant virions read through the plus and minus leader RNA termination sites very efficiently under standard transcription conditions in vitro (32, 33); a role for ATP is again indicated since the mutant viruses show much less dependence on the β-γ bond of ATP for initiation of RNA synthesis (36). Intriguingly, the properties of the polR virus seemingly result from a charged-amino-acid change in the N protein of their virion RNP templates (32).

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In this report, we explore in more detail the utilization of ATP by the wt and polR mutant VSV polymerase in vitro. For wt virus, we found that ATP concentration requirements differed between leader RNAs (plus or minus) and mRNAs. In contrast, polR mutants showed little or no difference in ATP requirements between these two types of transcripts. These results suggest that the ATP requirements for the transcription process may differ from those of the replication process and that this differential requirement manifests itself when the polymerase reaches the leader-N gene junction. The significance of these findings with regard to the putative role of virus protein phosphorylation in the regulation of VSV RNA synthesis is discussed.

MATERIALS AND METHODS

Growth, source, and purification of virus. Sources of wt standard VSV Indiana (Mudd-Summers strain), wt DI particles (DI 0.22, also called WT-T), and polR VSV mutants have been described elsewhere (32, 33, 35). Virus growth in BHK-21 cells and purification on sucrose and tartrate gradients were carried out as described previously (32, 34).

In vitro polymerase assay. All polymerase reactions were carried out in 100-μl volumes which contained 2 μg of standard virus or 10 μg of DI particles, 50 mM Tris-acetate (pH 8.0), 8 mM magnesium acetate, 0.3 M potassium acetate, 0.1% Nonidet P-40, 2 mM dithiothreitol, 100 μCi of labeled precursor per ml, and trace amounts of glycerol; fixed nucleotide substrate concentrations were maintained at 1 mM, except for the labeled precursor, which was adjusted to 0.1 mM for [^{3}H]CTP or [α-^{32}P]GTP and 0.03 mM for [^{3}H]UTP. All labeled precursors were obtained from ICN Pharmaceuticals, Inc., and unlabeled ones were obtained from Calbiochem-Behring. Incubations were carried out for 1 h at 30°C, and reactions were stopped by immersion in a dry ice-ethanol bath. Incorporation of label was measured by spotting duplicate 40-μl samples onto Whatman DEAE-81 filters, washing the filters, and counting the radioactivity as described previously (32). Under all conditions, at least 95% of the limiting substrate remained unincorporated.

Gel analysis of RNA products. Transcription reaction samples were treated with proteinase K, extracted with phenol-chloroform, spun through G-50 Sephadex to remove unincorporated label, and precipitated with ethanol as described previously (32). Analyses of glyoxylated RNA products on agarose gels, unmodified transcripts on 12% polyacrylamide–7 M urea gels, or RNA duplexes on nondenaturing 12% polyacrylamide gels were described previously (32).

End labeling and nuclease protection assay. End-labeling of purified standard wt virus RNA with cytidine 3′, 5′-bis-[^{32}P]phosphate and RNA ligase was carried out as described previously (32, 38). Annealing reactions were carried out in 250-μl volumes with 230 ng of labeled probe and 13 ng of transcriptase products by first denaturing in 10 mM Tris hydrochloride (pH 7.7)–1 mM EDTA buffer at 100°C for 1 min, adjusting the reaction mixture to 0.6 M NaCl–0.01% sodium dodecyl sulfate and incubating at 70°C. Samples were removed at the times indicated for digestion with a mixture of RNases A and T1, and resistant counts were determined as described previously (32). Before being used in these experiments, the probe was characterized by hybridization to purified transcriptase products containing about a 10-fold molar excess of plus-strand leader. After subtraction of background resistant counts in the absence of added products, it was determined that at least 70% of the probe could be protected. Quantitation of transcripts complementary to the 3′ end of this probe was then carried out with an excess of probe.

RESULTS

Effect of nucleotide concentration on standard virion polymerase of wt and polR VSV mutants. We have reported previously that the polR VSV mutants can utilize AMP-PNP for initiation of RNA synthesis in vitro much more efficiently than wt virus can (36). Why initiation normally requires the β-γ bond of ATP or why this function is bypassed in the mutants remains unclear. These observations prompted us to look for possible differences in the kinetics of ATP utilization by wt VSV versus the polR mutants. Accordingly, we measured RNA synthesis catalyzed by detergent-disrupted virions as a function of ATP concentrations ranging from 31 to 500 μM. All activity values reported were obtained reproducibly (±10%) in several different experiments with several different virus preparations of each type and were obtained after 1 h of incubation, at which time products have been shown to accumulate at an approximately linear rate for both wt and mutant viruses (36).

We found a distinct difference in the behavior of wt versus polR1 VSV in vitro polymerase activity (Fig. 1). Whereas the plot of activity versus ATP concentration for the polR1 VSV mutant is a conventional hyperbolic curve, the wt VSV, surprisingly, showed a sigmoidal curve, or cooperativity, reminiscent of an allosteric phenomenon (Fig. 1A). The requirement for relatively high ATP concentrations (>500 μM) needed to attain near maximum activity with wt virus is consistent with previously reported findings; the sigmoidal response of detergent-disrupted wt virus was evident in one previous study (4), but it was not apparent or otherwise noted in other studies (8, 47). Conventional double-reciprocal plots of the data (Fig. 1B) revealed that the polR1 virus activity followed a normal Michaelis-Menten response, extrapolating to an apparent K_m of ~143 μM, whereas wt virus showed an upward-deflecting curve, making attempts to derive an apparent K_m inappropriate. We did, however, estimate the approximate concentration of ATP needed for half-maximal activity of wt virus from plots like that shown in Fig. 1A, but using higher concentrations of the substrate, and this value appears to be close to the apparent K_m value of 500 μM reported previously (47).

At 1 mM ATP, the polR1 mutant displayed roughly half the activity of the wt virus (0.02 versus 0.04 μmol of [^{3}H]UMP incorporated per mg of virus per h). In contrast, the mutant was two to three times more active than the wt virus at low ATP concentrations. The independently isolated polR2 mutant, which we have shown previously to behave similarly to polR1 for readthrough of the leader-N gene junction (32), also showed conventional Michaelis-Menten kinetics, with a slightly lower apparent K_m of ~100 μM (data not shown). We have also observed that wt virus of the VSV New Jersey serotype (Ogden strain) behaves similarly to our wt Indiana strain (unpublished data). In light of a previous report on the presence of ATPase activity in purified VSV (40), which could potentially affect our measurements of ATP utilization, we have looked for significant depletion of ATP under our transcription conditions and found none (unpublished observations). We therefore conclude that the mutational change(s) in the polR VSV mutants affect the utilization of ATP by conferring a lower ATP requirement on the mutant polymerase reaction, thus eliminating the apparent cooperative effect observed with wt virus.

We next asked whether the kinetic differences between wt and polR VSV are unique to ATP utilization or whether

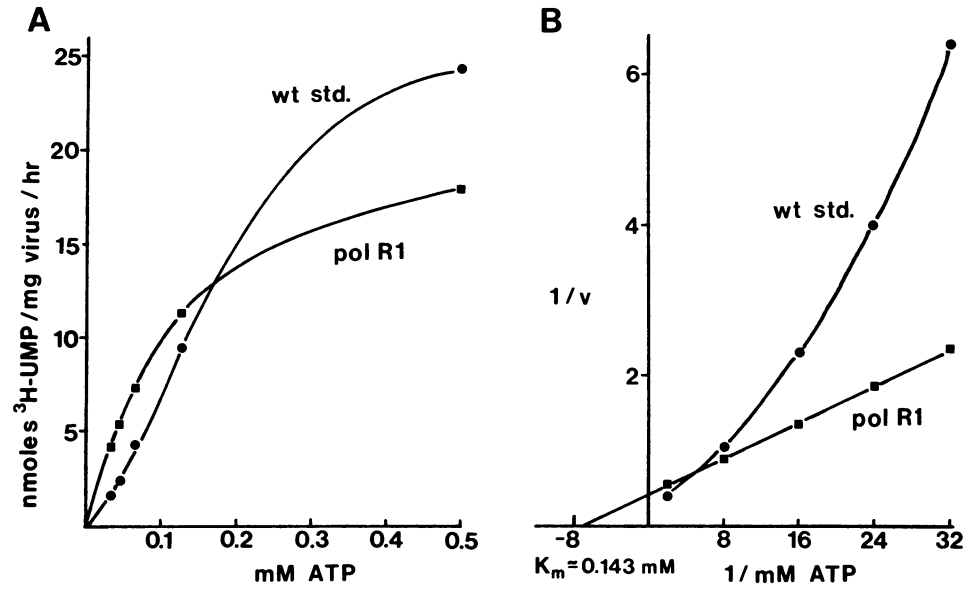

FIG. 1. Kinetics of ATP utilization by VSV wt and mutant polR1 standard virion polymerase in vitro. The assay conditions are described in the text. (A) Direct plot of activity versus ATP concentration. (B) Double-reciprocal plot of the same data. The activities in this panel are expressed as nanomoles of [^{3}H]UMP incorporated per 0.1 mg of virus per h.

other nucleoside triphosphate substrates are similarly affected. The kinetics of utilization of GTP, CTP, and UTP are shown in Fig. 2 as double-reciprocal plots of activities measured in parallel experiments. We observed small but significant differences between the mutant and wt virus; these differences were clearly not as dramatic as those seen with ATP. In all instances, the concentration requirements for half-maximal activity were below 50 μM, in agreement with a previous report (47). With wt virus, all three nucleoside triphosphates showed slight deviations from Michaelis-Menten behavior, again making it inappropriate to determine apparent K_m values by extrapolation. With GTP, but not CTP or UTP, the plot for the polR1 mutant was linear, with an apparent K_m of ~11 μM. The mutant was also significantly more active than the wt virus at low GTP and UTP concentrations. We conclude that the polR mutation(s) also affects utilization of other nucleoside triphosphates besides ATP but the differences are less striking. Because previous findings have suggested that ATP plays a unique role in VSV polymerase activity, we chose to further investigate the effects of this nucleotide.

In vitro transcript sizes as a function of ATP concentration. One possible effect of limiting nucleotide substrate concentrations in a polymerase reaction is premature termination. Therefore, the enhanced activity of the polR mutant at low ATP concentrations relative to that of wt virus might reflect a tighter association between the polymerase and its template. We would then expect a relative increase in the amounts of abortive products smaller than full-size mRNAs under limiting ATP conditions for wt virus. However, this was not the case (Fig. 3). Most of the wt virus transcripts synthesized under standard high-ATP conditions (Fig. 3,

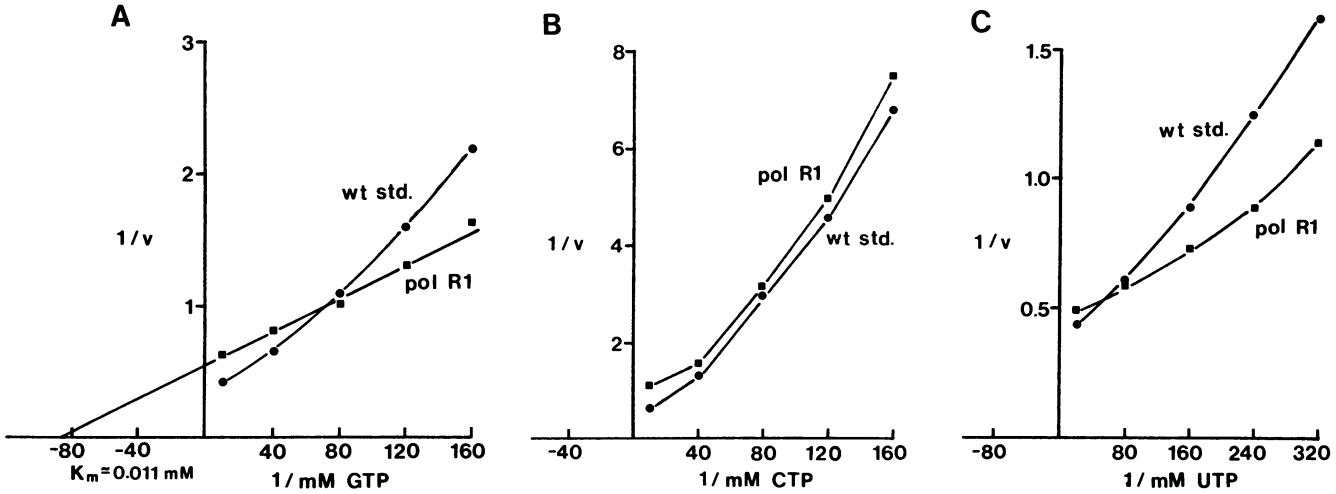

FIG. 2. Double-reciprocal plots of kinetics of utilization of GTP (A) CTP (B), and UTP (C) by wt and mutant polR1 VSV standard virion polymerases in vitro. The assay conditions were as described for Fig. 1 except that [^{3}H]CTP was used in place of [^{3}H]UTP for panel C.

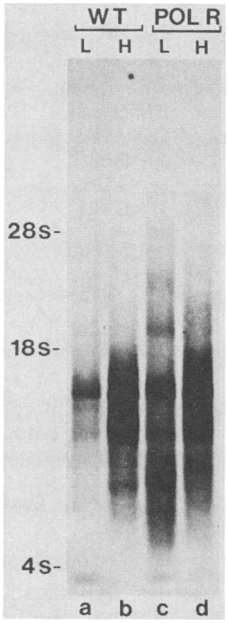

FIG. 3. Agarose gel analysis of glyoxal-denatured, [^{3}H]UMP-labeled polymerase transcripts from wt (lanes a and b) and polR1 VSV (lanes c and d) standard virions. Lanes L and H, Low-ATP (31 μM) and high-ATP (500 μM) reactions, respectively, carried out as described for Fig. 1. The positions of marker RNAs from mouse L cells run in a parallel lane are indicated (28S and 18S rRNAs, 4,712 and 1,869 bases long, respectively; 4S tRNA, ~75 bases long).

lane b) showed a size profile similar to those obtained in previous studies (34, 36). Because of the attenuated nature of VSV transcription, most of these products correspond to broad bands of N, NS, and M mRNAs, which are 1,326, 815, and 831 residues long, respectively; they appear diffuse because their poly(A) tails are of different sizes. Only one sharp band can be discerned (more clearly seen with shorter exposures), and this corresponds to a fraction of N transcripts which lack poly(A) tails, as previously described (34, 36). At a low ATP level (Fig. 3, lane a), most of the products also showed sizes expected for N, M, and NS mRNAs but the most prominent band corresponds to nonpolyadenylated N mRNA. Clearly, limiting ATP concentrations does not lead to a major shift toward smaller transcripts. The relative increase in nonpolyadenylated N mRNA was not observed in other experiments, and its significance is dubious. Note, however, the relative increase in the small amounts of leader-size transcripts which migrated ahead of the 4S tRNA marker at a low ATP concentration. Quantitation of plus-strand leader RNA, which represents only 3 to 6% of the total mass of products under standard conditions, is described below.

As shown previously (32, 34, 36), the polR1 transcripts synthesized under standard high-ATP conditions were generally similar to those of wt virus, except for somewhat more heterogeneous poly(A) tails and the presence of variable-size readthrough leader-N gene products which migrated between the leader RNA and the NS and M mRNAs (Fig. 3, lane d). The size profile of the polR1 products at a low ATP concentration (Fig. 3, lane c), however, was in general similar to that at a high ATP concentration (lane d), except for an increase in the small proportion of transcripts migrating slower than the 18S rRNA marker.

Although abortive transcription, or failure to complete elongation to full-size mRNAs, clearly does not explain the unexpectedly low wt virus polymerase activity at limiting ATP concentrations, we considered the possibility that increased attenuation at gene junctions might play a part in this phenomenon. If this was the case, a substantial decrease in the ratio of the comigrating NS plus M mRNAs to N mRNA in the wt virus products would be expected, since the N gene is situated upstream on the genome. However, quantitation by densitometric scanning of gel radioautographs similar to those shown in Fig. 3 indicated only a slight reduction in the ratio of NS plus M mRNAs to N mRNA (~10% at 62 μM ATP). We therefore conclude that the small increase in attenuation between the structural gene junctions is not enough to fully explain the low activity of wt virus relative to polR1 virus at a low ATP concentration.

Effect of ATP concentration on VSV DI particle minus-strand leader RNA synthesis. Wt VSV DI particles contain the same complement of proteins as the standard virion, including identical polymerase proteins. However, the predominant product (>95%) of in vitro transcription is the 46-nucleotide minus-strand leader RNA copied from the 3′ end of the DI template (25, 31, 32). If the low activity of standard wt virus at limiting ATP concentrations reflects a reduced ability to initiate transcription at the 3′ end of the template, a similar reduction in synthesis of minus-strand leader RNA from DI templates might be expected. Surprisingly, however, ATP utilization by DI particles followed Michaelis-Menten kinetics, with extrapolation to an apparent K_m of ~133 μM (Fig. 4a), which is close to that obtained with the standard polR1 virus (see above).

GTP and CTP utilization by wt DI particles also followed normal Michaelis-Menten behavior, with an apparent K_m of ~29 and ~43 μM, respectively (Fig. 4b and c). The kinetics of UTP utilization were not measured because a truncated form of minus-strand leader RNA is synthesized in the absence of this nucleotide substrate (the first U is at position 21) (42, 43). This is not a problem for standard virus products (Fig. 2C) because plus-strand leader represents only a small fraction (3 to 6%) of the total. We conclude that minus-strand leader transcription by wt VSV DI particles shows distinct kinetic differences in response to nucleotide substrate concentrations, compared with total wt standard virus products. The differences are particularly striking for ATP since the apparent cooperativity seen for standard virus products is not a feature of DI particle minus-strand leader RNA synthesis. PolR1 virus DI particles, which we have shown previously to synthesize an abundance of readthrough products (32, 33), also showed Michaelis-Menten behavior as a function of ATP concentration, with an apparent K_m similar to those of wt DI particles and polR1 standard virus (data not shown).

As with standard virus, no major change in the size of the wt DI product occurred at low (Fig. 5B, lane a) versus high (lane b) ATP concentrations. The multiplicity of bands close to the 47-nucleotide minus-strand leader RNA presumably reflects inexact termination and has been observed in a previous study (43). More importantly, low ATP levels clearly do not lead to a significant increase in readthrough at this junction.

Effect of ATP concentration on plus-strand leader RNA synthesis and lack of effect on readthrough of the leader-N gene junction. The results obtained with DI particles led us to hypothesize that the standard virus plus-strand leader, which, as mentioned above, represents 3 to 6% of the total products under standard conditions, might also behave like the minus-strand leader RNA in response to various ATP

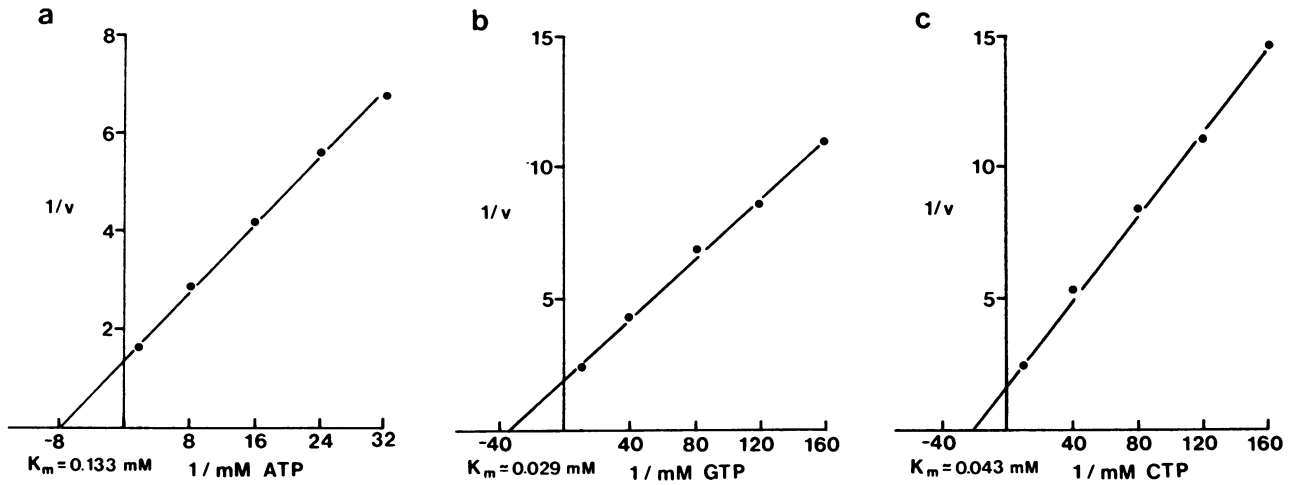

FIG. 4. Double-reciprocal plots of kinetics of ATP (a), GTP (b), and CTP (c) utilization by wt VSV DI particles. The assay conditions were as described for Fig. 1. Activities correspond to nanomoles of [^{3}H]UMP incorporated per mg of virus per h.

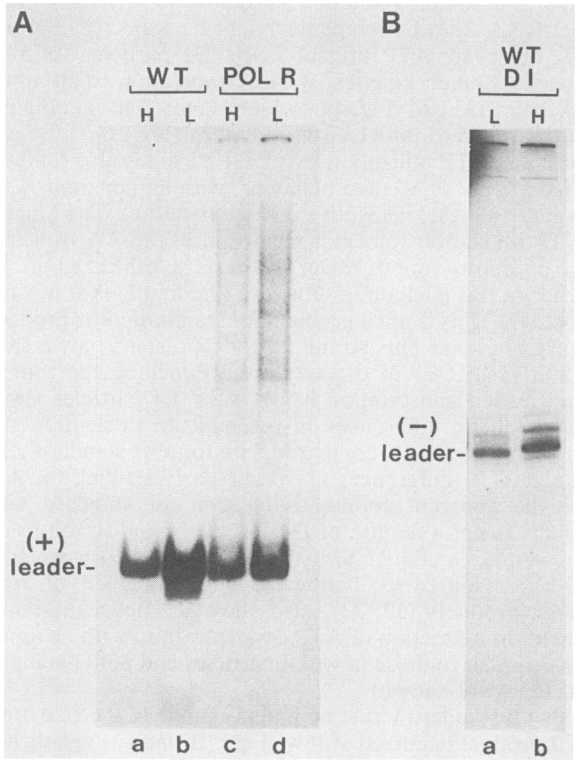

FIG. 5. (A) Acrylamide gel analysis of RNase-resistant, ^{32}P-labeled RNA duplexes after annealing of 3′-end-labeled genomic probe to wt and polR1 polymerase products. Lanes H and L, High-ATP (500 μM) and low-ATP (31 μM) reactions, respectively (corresponding to the samples in Fig. 6, after 6 h of annealing). Purified duplexes were electrophoresed on 12% nondenaturing acrylamide gels. (B) Acrylamide gel analysis of wt DI particle polymerase products. Lane a, Low ATP (62 μM); lane b, high ATP (1 mM). Reactions were carried out as described for Fig. 4, and purified transcripts were analyzed on 12% acrylamide–7 M urea gels.

concentrations. To test this hypothesis, we attempted to measure plus-strand leader RNA directly by separating total ^{32}P-labeled products on high-percent acrylamide gels, cutting out the presumed leader band, and determining Cerenkov counts. Although these experiments revealed a reproducible enrichment for the presumed leader band in products from low-ATP reactions, we could not determine a reliable curve for several reasons. First, the steps involved in the extraction and purification of separate RNA samples (see Materials and Methods), each corresponding to a reaction carried out at a different ATP concentration, resulted in variable scatter of data points. Note that all of the values reported for the previous experiments relied on spotting accurately measured, duplicate reaction sample volumes directly onto DEAE paper. Second, we could not assume, a priori, that plus-strand leader followed conventional Michaelis-Menten kinetics. Attempts to deduce a K_m by a best fit to the scattered data points were therefore not justifiable. Third, although plus-strand leader RNA was usually the major species in reaction products from standard virus in the size range of 47 nucleotides, some size heterogeneity, possibly representing termination at neighboring sites, was almost always observed (see Fig. 5B for similar size heterogeneity of minus-strand leader). Fourth, the possible presence of similar-size transcripts originating from internal initiations at the N, NS, and M genes, as documented by others (48), could not be disregarded.

We therefore restricted our approach to resolving whether plus-strand leader is indeed enriched in products obtained at a low ATP concentration by using a specific hybridization probe. Accordingly, we measured plus-strand leader synthesis at low (31 μM) and high (500 μM) ATP concentrations by annealing total products to an excess of a 3′-end-labeled genomic RNA probe as described previously (32). Under these conditions, the percentage of labeled probe resistant to RNase is a direct measure of the number of molecules initiated from the 3′ end of the standard genomic template. By determining the size of the RNase-resistant duplexes, the amounts of leader and readthrough products can be quantitated. Using this methodology, we have shown previously that the wt virus polymerase terminates synthesis at the leader-N gene junction for ~87% of these 3′-end-initiated molecules under standard high-ATP transcription conditions, whereas the rest correspond to readthrough and

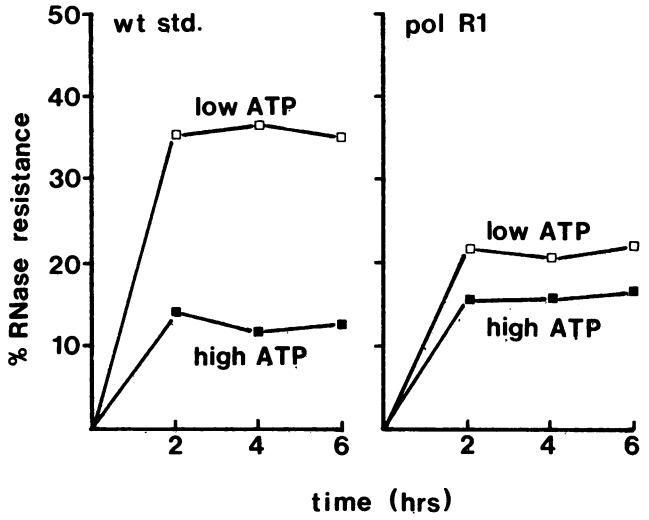

FIG. 6. Quantitation of plus-strand leader RNA and readthrough products by RNase protection of 3'-end-labeled genomic probe. The probe was annealed to wt and polR1 VSV polymerase products from low-ATP (31 μM) and high-ATP (500 μM) reactions carried out as described for Fig. 1. Preparation of the probe and assay conditions are described in the text.

termination at various sites within the N gene. Under the same conditions, polR1 virus synthesizes ~20% leader and ~80% readthrough transcripts, ranging in size from ~300 to ~800 bases and terminating heterogeneously within the N gene (32).

The proportion of 3'-end-initiated molecules from wt virus, relative to total products, was approximately 2.8 times more at a low ATP concentration than at a high one (Fig. 6). In contrast, polR1 virus showed only about 1.2 times more in the same comparison. Gel analysis of RNase-resistant duplexes obtained from reactions at high ATP levels confirmed our previous findings regarding synthesis of mostly leader-size molecules for wt virus and leader plus readthrough transcripts for polR1 virus (Fig. 5A). More importantly for this study, quantitation of the RNase-resistant duplexes (Fig. 5A) by densitometry showed only a small increase (~10 to 15%) in the relative proportion of readthrough to leader in low-versus high-ATP reactions. This indicates that replication may be slightly favored over transcription at a low ATP concentration, but the effect on antitermination is too small to imply a direct connection.

On the basis of these results, we conclude that synthesis of wt virus plus-strand leader RNA is not impaired to the same extent as that of mRNAs at a low ATP concentration. In fact, judging from the activity curve shown in Fig. 1A and the enrichment factor shown in Fig. 6, the sigmoidal response or cooperativity of total wt standard virus products is either absent or very much reduced for plus-strand leader RNA. Although for the technical reasons mentioned above, we were not able to determine a K_m value for the synthesis of plus-strand leader RNA, it is nonetheless clear that this leader species behaves more like minus-strand leader than wt mRNAs, insofar as both leaders appear to follow or approximate Michaelis-Menten kinetics, whereas wt mRNAs respond cooperatively.

On the basis of the results shown in Fig. 5A and 6, we infer that synthesis of polR1 virus leader RNA and readthrough transcripts also approximates Michaelis-Menten kinetics, with K_m values close to that of total polR1 products (~143 μM), because their proportion relative to the total RNA synthesized increased only slightly (~1.2-fold) at a low versus high ATP level. Lastly, the size analysis of RNase-resistant transcripts (Fig. 5A) revealed that readthrough of the leader-N gene junction was not affected by changes in ATP concentration for wt virus and only slightly for polR1 virus.

If it is assumed that the currently favored model for VSV transcription is correct and that plus-strand leader RNA synthesis must precede that of downstream mRNAs, our findings regarding higher ATP level requirements for wt mRNA synthesis than for leader RNA synthesis are in apparent conflict with results of a previous study by Testa and Banerjee (47). These investigators reported that VSV cores, free of virus envelope components, required less ATP (K_m ~50 μM) for elongation than for initiation (K_m ~500 μM). Elongation requirements were measured after a preinitiation step in the presence of high concentrations of ATP and CTP, which permits synthesis of the first phosphodiester bond of the leader RNA. This preinitiation step could conceivably be responsible for the lower ATP requirements these investigators observed for subsequent mRNA synthesis. Moreover, we have also observed that removal of viral envelope components from wt virus, but not polR virus, does affect the ATP response (J. D. Beckes and J. Perrault, manuscript in preparation).

DISCUSSION

The major findings reported here involve two distinct but related observations. First, the ATP concentration requirement for synthesis of wt VSV leader RNAs differs from that for mRNA synthesis. The latter clearly displays cooperativity with respect to ATP concentration. For the most part, the sizes of the products were similar over the range of ATP concentrations tested, and half-maximal activity was reached at ~500 μM. In contrast, minus-strand leader synthesis from wt DI particles followed Michaelis-Menten kinetics, with a K_m value of ~133 μM. Although we could not directly determine a K_m value for wt plus-strand leader RNA synthesis, we nonetheless clearly showed that this transcript also does not respond in the cooperative manner of wt mRNAs, since it was considerably enriched (~2.8-fold) in products obtained under low-ATP conditions. It is possible that both plus- and minus-strand leader RNA syntheses share identical K_m values for ATP, but this remains to be determined.

Whether the kinetic behavior of wt mRNAs reflects an additional regulatory site(s) on the polymerase, i.e., an allosteric phenomenon, or the combined effect of two or more different ATP-requiring reactions cannot be inferred solely from our results. One possibility is that two separate template-polymerase complexes are involved, one for leader and one for mRNA, as suggested previously on the basis of inhibitor studies (46). Alternatively, the activity of the VSV template-polymerase complex may depend on a modification which is required for mRNA synthesis but not, or less so, for leader RNA synthesis. Either the modification itself or the resulting modified template-polymerase complex could then be viewed as requiring the higher ATP levels. Whatever the explanation, it is clear that leader RNA synthesis can be partially uncoupled from mRNA synthesis in vitro solely by varying the ATP concentration.

Our second major observation was that the polR VSV mutants displayed a change in their ATP concentration requirements such that the total mass of products followed a

Michaelis-Menten response with a K_m similar to that of minus-strand leader RNA synthesis from wt DI particles. It should be recalled here that these mutant viruses have a unique phenotype in vitro: in addition to synthesizing leader RNA and mRNAs, the polymerase reads through the leader termination sites efficiently and yields substantial amounts of abortive replicationlike products (terminating heterogeneously within the N gene) (32). As with wt standard virus, the sizes of the mutant transcripts were similar over the range of ATP concentrations tested. Mutant plus-strand leader RNA and readthrough products, however, were only slightly enriched (~1.2-fold) in low-ATP reactions, and the ratio of readthrough transcripts to leader was also only slightly affected (~15 to 20% increase). All mutant transcripts must therefore lack the ATP cooperative response characteristic of wt mRNAs and thus approximate Michaelis-Menten kinetics, with K_m values similar to that for wt minus-strand leader RNA. Why this is so is not clear, but it may be due to differences in protein phosphorylation (see below).

It is important to note that the polR virus mutational change in ATP dependence correlates with the synthesis of readthrough products; this suggests a link between the two. It may well be that synthesis of plus-strand leader from standard genomic templates and minus-strand leader from DI templates represent analogous replication initiation events which are independent of, or less dependent on, the ATP-requiring function responsible for the cooperativity phenomenon seen with wt mRNAs. The cooperative response with ATP, perhaps reflecting the need for additional phosphorylation, may therefore be specific to the normal transcription process and not the replication process. Lowering the ATP concentration, however, does not by itself lead to a major change in termination. The exact relationship between these parameters thus remains elusive.

We see several reasons why the uncoupling of leader RNA and mRNA synthesis, as well as the switch from transcription to replication, is likely to involve virus protein phosphorylation. First, the β-γ bond of ATP is required to initiate wt virus RNA synthesis in vitro and, if this step is bypassed by preinitiation, elongation in the presence of the analog AMP-PNP leads to complete readthrough synthesis (49). Second, the polR mutants also show a much reduced dependence on the high-energy bond of ATP for initiation of RNA synthesis in vitro (36). We have not yet determined whether the high ATP requirements specific for wt virus mRNA synthesis documented here also depend on the β-γ bond of ATP, but this would not be surprising. In any case, the two ATP requirements are most likely related, inasmuch as the two independently isolated polR mutants show changes in both. Third, De and Banerjee (14) have recently shown that the ratio of plus-strand leader to N mRNA increases when the virus NS phosphoprotein, but not the L polymerase protein, is limiting during transcription under standard high-ATP conditions. Since it has been established that only more highly, and specifically, phosphorylated species of NS protein are active in reconstituting polymerase activity with purified L protein and the RNP template (3, 10), the ATP cooperativity effect we observed here could reflect a more critical need of this specific phosphorylation for synthesis of wt mRNAs as opposed to leader RNAs. Fourth, we have recently uncovered significant differences in protein phosphorylation between wt and polR mutant viruses (Beckes and Perrault, in preparation).

Several studies over the last decade have suggested that the VSV particle-associated protein kinases are cellular in origin (12, 21, 23, 29, 44). Recently, however, very strong evidence for a virus-specified protein kinase, copurifying with L polymerase protein, has been reported (10, 19, 27, 41). So far, only the phosphorylation of two serine residues in domain II of the NS protein has been linked to transcription activity in vitro (10). In view of this, it is surprising that polR VSV mutants carry out extensive transcription in the presence of AMP-PNP in place of ATP (36) since these conditions should not allow phosphorylation of the critical serines. Perhaps the mutant virus NS proteins acquire the proper phosphorylation state in vivo before virus release. Alternatively, the specific phosphorylation may normally modulate an interaction with other components of the template-polymerase complex; the mutational alteration in polR could then be viewed as compensatory and substitute for the missing phosphorylation event.

Although we have presented arguments for linking phosphorylation of viral proteins to the differing ATP requirements for polymerase activity observed in this study, it must be noted that the kinase activities associated with VSV particles, including the one copurifying with the L protein, display relatively low ATP concentration requirements (<100 μM) for near maximal activity (23, 27). However, even when phosphorylation of the two viral NS protein subspecies was studied under transcription reaction conditions (27), only autoradiographs of labeled bands were presented and no K_m values could be derived from the data. Phosphorylation at specific sites within the NS proteins or some other viral protein(s) could conceivably require higher concentrations of ATP. For example, autophosphorylation of the $pp60^{v\text{-}src}$ protein involves additional sites when the concentration of ATP is high, even though the K_m value appears to be low (39). More detailed studies of the ATP requirements of the VSV-associated protein kinases, currently under way in our laboratory, will hopefully shed light on this question.

We have also considered the possibility that a high ATP concentration requirement for poly(A) addition to VSV mRNAs might explain the differing ATP responses documented in this report. However, we do not think this is very likely. Leader RNAs, in contrast to mRNAs, lack poly(A), but Fig. 3 clearly shows that a low ATP concentration did not lead to a major increase in nonpolyadenylated mRNA transcripts or to an increased attenuation between the major structural gene junctions for either wt or mutant standard viruses. Therefore, the major effect of low ATP concentration for wt standard virus is, for the most part, specific to the junction between the plus-strand leader gene and the N gene, not at sites for poly(A) addition. Furthermore, it seems probable that the change in ATP utilization between mutant and standard viruses is related to the changes in requirements for the β-γ bond of ATP, as mentioned above. A high ATP concentration requirement for poly(A) addition would not explain the need for this bond.

Whatever the nature and complexity of the ATP reactions involved in regulating VSV RNA synthesis, it is intriguing that the changes in the polR mutants may all be due to one mutation in their RNP core-associated N protein (32). So far, we have no evidence that any other viral protein carries a relevant mutation. The readthrough property of these mutants is tightly associated with the RNP core, and not the L and NS proteins (32), and so is the reduced dependence on the β-γ bond of ATP for initiation of synthesis (Beckes and Perrault, in preparation). We have proposed previously that RNP template conformational changes could govern the balance between transcription and replication (32, 37). It

should be rewarding to elucidate the putative role played by the N protein in affecting ATP requirements for VSV RNA synthesis.

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