Altered ATP Function of a Vesicular Stomatitis Virus Mutant Detected by Kinetic Analysis of the Transcriptase Using Phosphorylated Ribavirin

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SUMMARY

We have studied the effect of phosphorylated ribavirin on the vesicular stomatitis virus (VSV) in vitro polymerase reaction by analysis of kinetic data obtained by varying the concentration of nucleoside triphosphates. The wild-type VSV had previously shown a competitive inhibition with the four natural nucleoside triphosphates with the use of ribavirin diphosphate (RDP) or ribavirin triphosphate (RTP). In contrast, when RDP (or RTP) was added to a transcription assay system using the polR1 mutant of VSV, a non-competitive or mixed type of inhibition was observed when the concentration of ATP was varied. Our results indicate that polR1 has an altered ATP function in addition to the previously described phenotypic characteristics of this mutant, which include synthesis of readthrough products of the leader/nucleocapsid (N) gene junction and a decreased ATP requirement for transcription. We have also studied CsCl-purified in vitro transcription products by primer-extending leader or N mRNA transcripts and found that the ratio of leader/N mRNA for VSV polR1 (1.3:1) was lower than values obtained previously for wild-type (3.7:1).

Vesicular stomatitis virus (VSV) is the prototype rhabdovirus containing a negative-stranded RNA genome 11 kb long. The transcription and replication events of this virus have been studied for several years, but many questions related to these processes and to the switch from the transcriptive to the replicative mode remain unanswered. The VSV polR mutants (Perrault et al., 1983) have unique properties that may help solve the intricacies of transcription and replication. The properties of the polR mutants include synthesis of leader/nucleocapsid (N) gene readthrough transcripts (Perrault et al., 1983), utilization of imido-ATP for transcription initiation (Perrault & McClear, 1984), and an altered ATP utilization (Helfman & Perrault, 1988), whereas wild-type (wt) VSV has a high ATP requirement as well as an obligatory cleavage of the beta-gamma bond of this nucleotide for transcription (Testa & Banerjee, 1979; Green & Emerson, 1984; Perrault & McClear, 1984). We have used ribavirin to investigate by enzyme kinetic procedures and product analysis the mechanism of VSV mRNA synthesis, in an effort to understand the in vitro transcription of the virus.

We have already reported that ribavirin 5'-monophosphate, 5'-diphosphate (RDP) and 5'-triphasphate (RTP) possess a significant direct suppressive effect upon viral polymerase activity. Inhibition by RDP or RTP could be reversed by addition of GTP, CTP and UTP, but not by the addition of GDP or ATP (Toltzis et al., 1988). The effective reversal of inhibition by all nucleoside triphosphates except ATP suggested that the ribavirin molecule does not act only as a guanine analogue as generally believed, but rather may interact with the polymerase complex on a site specific for at least three of the precursors of RNA synthesis. The fact that the effect of phosphorylated ribavirin could not be reversed with the addition of ATP could be explained if ATP had an additional interaction with the polymerase complex, at a site other than the polymerization site.
The mechanism of inhibition of the VSV polR1 transcriptase by phosphorylated ribavirin compounds was studied by varying the concentration of the four nucleoside triphosphates. We have reported elsewhere that RDP and RTP are selective inhibitors of the VSV wt transcriptase in vitro, and that this inhibition is competitive with all four nucleoside triphosphates (Fernandez-Larsson et al., 1989). We report here that the polR1 mutant virus, however, showed a mixed-type non-competitive inhibition with ATP.

Viruses and their source were as follows: VSV (Indiana serotype) was from stocks maintained at this laboratory; VSV polR1 was kindly sent to us by Dr Jacques Perrault. Both viruses were purified in sucrose gradients after growth in Chinese hamster ovary cells as described by Stampfer et al. (1969). VSV wt was grown at 34 °C for 18 to 20 h, and polR1 was grown at the same temperature for 27 to 30 h. RDP and RTP were a kind gift of Roland K. Robins and Daniel Smith (Allen et al., 1978).

The in vitro transcription reactions were performed as previously described by Banerjee (1981). The reaction mixture was composed of 100 mM-NaCl, 50 mM-Tris–HCl pH 8.0, 5 mM-MgCl₂, 4 mM-dithiothreitol, 0.05% Triton X-100, 10 units of placental RNase inhibitor (Boehringer Mannheim), 20 μCi [³²P]UTP or [³²P]ATP (Amersham) and 25 to 50 μg of VSV virions, in a total volume of 0.2 ml. Nucleoside triphosphate precursors (Boehringer) were adjusted to 0.1 mM for GTP, CTP and UTP, and to 1 mM for ATP, except for the one that was varied, and the reaction mixtures were incubated for 60 or 90 min at 30 °C. The concentration of RDP or RTP was adjusted to 0.15 mg/ml in the reaction tubes that contained inhibitor. The incorporation of ³²P was measured by trichloroacetic acid precipitation and counted by liquid scintillation. Samples were counted at time zero to determine background. Linear regression lines for plots were calculated using the computer program Sigma-Plot (Jandel Scientific).

Whereas the intersection of the lines of double-reciprocal plots are well defined in the case of competitive and non-competitive types of inhibition, a mixed inhibitor is graphically defined as one that causes the reciprocal plot lines to intersect in the left upper quadrant, except on the 1/[S] axis (Dixon & Webb, 1979). Our experiments gave this 'mixed-type' of inhibition for polR1 when the concentration of ATP was varied using RDP (Fig. 1a) or RTP (Fig. 2a), while the inhibition was of the competitive type when the other three phosphorylated nucleosides were varied individually with RDP (Fig. 1a) or RTP (data not shown) as the inhibitor. This is in contrast to results obtained with the wt VSV, where RDP (Fig. 3a) or RTP (Fig. 4a) gave a competitive type of inhibition when [ATP] was varied.

The same interpretation of the data resulted when we used Eadie-Hofstee plots of V₀ against V₀/[S] for the polR1 (Fig. 1b and 2b) and the wt VSV data (Fig. 3b and 4b). Therefore, we conclude that this type of inhibition is consistent with our findings.

Kinetic constants were calculated using a statistical method (Wilkinson, 1961) (Table 1). The $K_m$ values obtained for the four nucleoside triphosphates without inhibitor are similar to those reported for polR1 (Testa & Banerjee, 1979; Beckes et al., 1987).

The leader region of VSV RNA has a direct role in binding the polymerase and in initiating transcription. Therefore, we investigated the possibility that the behaviour of the polR1 polymerase and the apparently different leader/N mRNA ratio in vitro (see below) was related to an alteration in the sequence of the 3' end of the genome by sequencing the leader, the intergenic region and the first 70 nucleotides of the 3' end of the N gene. To sequence the leader RNA transcript and the 5' end of the N mRNA directly by the deoxyxynucleotide chain termination procedure (Sanger et al., 1977) we used reverse transcriptase and two specific oligonucleotide primers described below. We used a primer complementary to genomic-sense leader to sequence the intergenic region directly from genomic RNA. We found that the first 120 nucleotides of the 3' end of the polR1 genome were identical in sequence to the VSV wt (data not shown).

We are reporting elsewhere that the inhibitory effect of ribavirin on the VSV wt polymerase is at the level of primary transcription, when the transcriptase enters the 3' end of the genome. We therefore wanted to know whether the phosphorylated ribavirin compounds had a similar effect on the in vitro polR1 transcription, given the altered ATP function of this virus. In vitro transcription reactions were terminated and the products were solubilized with uninfected cellular extracts in NP40 lysis buffer (0.14 M-NaCl, 1.5 mM-MgCl₂, 10 mM-Tris–HCl pH 8.6,
Fig. 1. Effect of nucleoside triphosphates on the kinetics of inhibition by RDP. VSV polR1 mutant RNA polymerase activity was determined by measuring $[^{32}P]UTP$ incorporation at different concentrations of ATP, GTP and CTP in the presence (●) and absence (○) of RDP. The incorporation of $[^{32}P]ATP$ was measured when the concentration of UTP was varied. The concentration of RDP was 150 μg/ml. The data are presented as Lineweaver-Burk (a) and Eadie-Hofstee (b) plots.
Fig. 2 to 4. Effect of ATP on the kinetics of inhibition by RTP of polR1 mutant polymerase activity (Fig. 2), by RDP of wild-type polymerase activity (Fig. 3) and by RTP of wild-type polymerase activity (Fig. 4). Activities were determined and plots (a) and (b) are as in Fig. 1.
Table 1. Kinetic constants for nucleoside triphosphates with VSV polR1 RNA polymerase*

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<th>No inhibitor</th>
<th>RDP</th>
<th>RTP</th>
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<tr>
<td>ATP</td>
<td>98 ± 20†</td>
<td>203 ± 37</td>
<td>133 ± 23</td>
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<tr>
<td>GTP</td>
<td>22 ± 2</td>
<td>39 ± 4</td>
<td>32 ± 9</td>
</tr>
<tr>
<td>CTP</td>
<td>23 ± 2</td>
<td>75 ± 19</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>UTP</td>
<td>74 ± 0.5</td>
<td>26 ± 3</td>
<td>15 ± 5</td>
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* Polymerase activity was measured by incorporation of [32P]UTP when ATP, GTP and CTP concentrations were varied and by incorporation of [32P]ATP when UTP concentration was varied. Kinetic constants were calculated by directly fitting the data to hyperbolae as described by Wilkinson (1961).
† Expressed as \( K_m \) (μM) ± standard error.

0.5% NP40) as described previously (Belloq et al., 1987). In vitro transcription products synthesized in the presence or absence of RDP or RTP were purified by CsCl centrifugation as described by Leppert et al. (1979) and primer-extended with oligonucleotides specific for primer or N mRNA transcripts. The first primer was complementary to 15 nucleotides of leader RNA to give a 30-mer extended product, and the second primer was complementary to 15 nucleotides of nucleocapsid mRNA to give a 50-mer extended product. As controls for primer extension, VSV- and mock-infected cell RNAs were prepared as previously described (Leppert et al., 1979). The two specific primers, labelled at their 5' ends with [γ-32P]ATP and T4 polynucleotide kinase, were added to each of the RNA samples and extended with 16 units of avian myeloblastosis virus reverse transcriptase in a reaction mixture containing 0.5 mM each of dATP, dGTP, dCTP and dUTP, 80 mM-NaCl, 50 mM-Tris-HCl pH 8.3 and 8 mM-MgCl2 for 90 min at 42 °C. The products were phenol-extracted, ethanol-precipitated and analysed on 10% polyacrylamide gels (Sanger & Coulson, 1978) (Fig. 5). The extended products were excised from the gel after autoradiography and counted by liquid scintillation.

We have found that the ratio of leader to N mRNA for polR1 VSV (1.3 : 1) is lower than values obtained previously for wt VSV (2.9 : 1) (Fernandez-Larsson et al., 1989). This difference in ratios was expected, considering that polR1 synthesizes a high proportion of leader/N gene readthrough transcripts (Perrault et al., 1983). On the other hand, intracellular transcripts analysed in the same manner showed a similar ratio of leader to N for polR1 and wt viruses (1 : 2.1 and 1 : 2.4, respectively). Surprisingly, little information is available on ratios of leader RNA transcripts to other transcripts in the infected cell. Work of Iverson & Rose (1981) predicts that the amount of leader transcripts, although not analysed directly in their study, should be greater than the amount of N mRNA. Since our data on the intracellular ratio of leader to N mRNA are the steady state levels of leader and N mRNA rather than synthesis levels, our results are not necessarily contradictory to their hypothesis. It has been postulated that leader RNA turns over more rapidly than messages, perhaps due to the lack of a poly(A) tail. Furthermore, it is only late in infection when there is sufficient N protein that leaders become encapsidated and stabilize in the cytoplasm (Blumberg & Kolakofsky, 1981; Piwnica-Worms & Keene, 1985). Leader RNA transcripts are also known to localize in the nucleus of the infected cells (Kurilla et al., 1982) and therefore would not be accessible in our cytoplasmic extract preparation.

The altered ATP function observed in the kinetic analysis of the polR1 mutant is compatible with the observation discussed above for ATP reversibility. Both support the argument for an additional ATP-binding site on the polymerase complex. Two distinct ATP-binding sites have been previously identified in the L protein, but none were found in the NS or N proteins, the other two components of the polymerase complex (Massey & Lenard, 1987). It is striking, however, that the readthrough characteristics of the polR1 (Perrault et al., 1983) as well as its altered ability to utilize ATP (Helfman & Perrault, 1988) have been shown by reconstitution experiments to map to a mutation in the N protein and apparently not the transcriptase, and that the alteration of this N protein amounts to a single amino acid substitution (Helfman et al., 1988). An independently isolated polR2 virus, which has the same phenotypic characteristics as polR1, contains an identical single-site mutation in its N protein (J. Perrault, personal communication).
Fig. 5. Determination of the ratio between leader and N mRNA from transcripts purified from VSV polR1 \textit{in vitro} transcription reactions. RNA transcripts were purified through CsCl as described in the text. Lanes 1 and 2 contain primer-extended products from \textit{in vitro} transcribed reactions done in the presence of RDP; lane 2 has one-tenth the amount of RNA as the sample extended in lane 1. Lane 3 represents primer-extended products from reactions without drug. Intracellular RNAs from mock- (lane 7), VSV polR1- (lanes 4 and 5) and VSV wild-type-infected cells (lane 6) were primer-extended as controls for the reactions. Lane 5 has one-tenth the RNA extended in lane 4. Lane 8 contains M, markers.

We do not know whether the observations reported here are the result of the change in the nucleocapsid protein of polR1. Most probably, the effects of this or another mutation in the polR1 virus alter the kinetic behaviour of the polymerase complex and unmask the additional ATP-binding site that exists in the L protein of this virus, as well as in the wild-type. There is evidence that the kinetic properties of ATP are related to phosphorylation events essential for transcription; a number of these have been reported for VSV. Phosphorylation of the NS protein is essential for \textit{in vitro} transcription (Gill \textit{et al.}, 1986; Chattopadhyay & Banerjee, 1987). Specifically, two serine residues within domain II of the NS protein must be phosphorylated by the kinase associated with the L protein (Sánchez \textit{et al.}, 1985). They in turn may lead to modulation of transcription/replication. Further experiments are in progress by us and others to clarify which gene affects the mixed ATP kinetics, in an effort to understand the molecular details of VSV transcription.

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REFERENCES


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