
Key words: VSV/polyadenylation/L protein

The L Protein of Vesicular Stomatitis Virus Modulates the Response of the Polyadenylic Acid Polymerase to S-Adenosylhomocysteine

By D. MARGARET HUNT,*1, 2 ROSHNI MEHTA†2, AND KAREN L. HUTCHINSON1

1Department of Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, South Carolina 29208 and 2Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216, U.S.A.

(Accepted 22 June 1988)

SUMMARY

TsG16(I) is a temperature-sensitive (ts) mutant of vesicular stomatitis virus, Indiana serotype, which overproduces polyadenylic acid [poly(A)] in an in vitro transcription system due to a mutation in the L protein. Others have reported that L-S-adenosylhomocysteine (S-Ado-Hcy) causes wild-type (wt) virus to overproduce poly(A) in vitro. The possibility that tsG16(I) constitutively expresses a property induced by S-Ado-Hcy in the case of wt virus was found not to be so since polyadenylation by the mutant was still sensitive to S-Ado-Hcy. Indeed, S-Ado-Hcy caused tsG16(I) to overproduce poly(A) in vitro to a greater extent than its parental wt virus. The increase in polyadenylation observed in response to saturating levels of S-Ado-Hcy differed for tsG16(I), for its parental wt virus and for another wt strain. To characterize which viral protein modulated the polyadenylation response to S-Ado-Hcy, purified virions were fractionated and their phenotypes in homologous and heterologous reconstitution assays were examined. The results indicated that the viral L protein modulated the response in all three stocks of virus. These data provide further evidence to suggest that the L protein of vesicular stomatitis virus plays a role in polyadenylation of the viral mRNA.

INTRODUCTION

Vesicular stomatitis virus (VSV) is a non-segmented, negative-stranded RNA virus belonging to the family Rhabdoviridae. Virions contain an RNA-dependent RNA polymerase (transcriptase) (Baltimore et al., 1970) which transcribes the genome in vitro and in vivo to produce five monocistronic mRNAs (Banerjee, 1987). The template for transcription is the genomic RNA–viral N protein complex; two other viral proteins, L and NS, are also necessary (Emerson & Yu, 1975) and the viral M protein can modulate transcription (Banerjee, 1987). Viral mRNAs made in vitro from purified virions are capped, methylated [having the 5' terminal sequence m7G(5')ppp(5')Am] and polyadenylated; all these modification reactions are tightly coupled to transcription (Banerjee, 1987). The polyadenylic acid [poly(A)] polymerase probably synthesizes poly(A) by 'chattering' on a stretch of seven U residues which occur at the end of each protein-coding gene (Banerjee et al., 1974; Clerx-Van Haaster et al., 1980; Herman et al., 1980; McGeech, 1979; Schubert et al., 1980). It is not known whether the poly(A) polymerase is a virus or host-encoded enzyme, although there is evidence to suggest that it is tightly associated with the transcriptase complex, that transcription and polyadenylation are closely coupled events (Villarreal & Holland, 1973; Banerjee et al., 1974; Murphy & Lazzarini, 1974; Deutsch & Banerjee, 1979; Herman et al., 1980; Hunt et al., 1984) and that the L protein can modulate polyadenylation (Hunt et al., 1984).

*Present address: Plant Hormone Laboratory, USDA, ARS, Beltsville Agriculture Research Center, Beltsville, Maryland 20705, U.S.A.
We have been using temperature-sensitive (ts) mutants of VSV to investigate the nature of RNA synthesis by VSV and the role of viral proteins in RNA synthesis and modification. TsG16(I) is a complementation group I mutant which was isolated in Glasgow (Pringle, 1970b). It has been proposed that the ts phenotype of tsG16(I) is correlated with a thermolabile L protein (Hunt et al., 1976). We have previously reported (Hunt, 1983) that RNAs made at 31 °C in vitro by tsG16(I) contain the same methylated cap structures as RNAs made by wild-type (wt) virions and are translated as efficiently in a reticulocyte translation system, giving rise to proteins of identical Mr in similar ratios. However, the tsG16(I) transcripts possess abnormally long tracts of poly(A) which migrate on gels as if a high proportion were five to 10 times as long as poly(A) tracts made in vitro by wt virions. This aberrant polyadenylation by tsG16(I) is due to an altered L protein (Hunt et al., 1984) and is observed at all temperatures tested from 27 °C to 39 °C (Hunt, 1983; Hunt et al., 1984).

Rose et al. (1977) reported that aberrantly long tracts of poly(A) are made by wt VSV in the presence of L-S-adenosylhomocysteine (S-Ado-Hcy). It was thus possible that the excessive polyadenylation by tsG16(I) was due to a mutation that caused it to express constitutively an activity which was inducible by S-Ado-Hcy in wt virus. Therefore, we investigated whether tsG16(I) was as susceptible to the effects of S-Ado-Hcy as its parental wt, and report here that it was, suggesting that the effects of the mutation or the addition of S-Ado-Hcy on the activity of the poly(A) polymerase were not identical. Furthermore, in the course of these experiments we discovered that the extent of the increase in poly(A) synthesis by tsG16(I), its parental wt virus (VSV Indiana, Glasgow strain) and another wt virus (VSV Indiana, San Juan strain) in response to addition of S-Ado-Hcy was different for each virus. We made use of this to show that the L protein can modulate the response of the poly(A) polymerase activity to S-Ado-Hcy. This therefore provides further evidence to suggest that the L protein plays a role in poly(A) synthesis by VSV.

**METHODS**

**Virus.** The Glasgow strain of wt VSV (Indiana), ts mutants isolated in Glasgow and the San Juan strain of VSV (Indiana) were kindly supplied by R. R. Wagner and S. U. Emerson, Charlottesville, Va., U.S.A. who received the Glasgow parental strain and mutants from C. R. Pringle, then in Glasgow, U.K. and the San Juan strain from the U.S. Department of Agriculture, Beltsville, Md., U.S.A. For the ts mutants, the letter G indicates the that they originated in Glasgow and the roman numeral indicates the complementation group. Virus was grown and harvested as previously described (Hunt, 1983) and stored in 15% glycerol, 2 mM-EDTA, 10 mM-Tris-HCl pH 7-4 at −18 °C.

Virus was titrated as previously described (Hunt, 1983; Hunt et al., 1984). No wt revertants of the ts mutants were detected.

Viral protein concentrations were determined by the method of Bradford (1976) using reagents from Bio-Rad. **Template preparation.** The procedure for template purification was identical to that reported previously (Hunt et al., 1984) except that 1 mM-EDTA was included in all buffers.

L and NS protein purification. The procedure for preparation of L and NS proteins from purified virions was identical to that reported previously (Hunt et al., 1984) except that minor modifications were made according to the virus strain or mutant used. The first step, in which solubilized virions were stripped of most of the M and G proteins by gel filtration on Bio-Gel A-50m (Bio-Rad) columns, was as described except that in the case of wt fractionations 1 mM-EDTA was added to all buffers. The subsequent steps involving solubilization of L and NS proteins from the template, centrifugation to sediment the template, lowering the NaCl concentration of the supernatant to 0-117 M by gel filtration using Sephadex G-50 and then chromatography on phosphocellulose and DEAE-cellulose were carried out according to Hunt et al. (1984) for wt(San Juan) and tsG16(I). However, this procedure was unsatisfactory for wt(Glasgow) in that the L-containing fraction from the phosphocellulose column contained considerable amounts of NS protein. This contamination was reduced by including 1 mM-EDTA in the 0-117 M-NaCl column buffer (0-117 M-NaCl, 33% glycerol, 0-07% Triton X-101, 0-2 mM-dithiothreitol, 33 mM-Tris–HCl pH 7-4) used to elute the Sephadex G-50 column and to equilibrate the phosphocellulose column.

**Transcription assays.** The transcriptive activity of solubilized virions (150 μg viral protein/ml reaction mixture) or of reconstitution mixtures containing subviral fractions in various combinations was determined as described by Hunt et al. (1984). Transcription assays contained 700 μM-ATP, 700 μM-GTP, 700 μM-CTP and 60 μM-UTP, plus 10 μCi [3H]UTP/ml, 100 μCi [3H]ATP/ml, or 5 to 10 μCi [α-32P]UTP/ml as required and did not contain a methyl donor. Reactions were carried out at 31 °C (the permissive temperature for the ts mutants). The results reported are those for the 120 min time points. We observed no significant differences in the extent of
polyadenylation at 60 min compared to 120 min (data not shown). Incorporation of radioactivity into ice-cold TCA-precipitable material was determined as described (Hunt, 1983; Hunt et al., 1984). The extent of polyadenylation was assayed by measuring the ratio of pmol of AMP relative to UMP incorporated in a transcription mixture which included both [3H]ATP and [α-32P]UTP (Hunt et al., 1984). The excess incorporation of AMP compared to UMP was used as a measure of polyadenylation. For example, if an RNA is 1600 nucleotides long with a poly(A) tail of 160 A residues, then it would be expected to contain approximately 400 residues of UMP or 560 (400 plus 160) residues of AMP when the poly(A) tail is taken into account. Thus the AMP : UMP ratio will be 1:4:1. If the poly(A) tail were to increase to 320 nucleotides, the AMP : UMP ratio would be 1:8:1. Regardless of the initial length of the poly(A) tail for the RNA used in this example, an increase of 160 nucleotides in the poly(A) tail length will cause an increase of 0-4 in the AMP : UMP ratio. If the results were expressed as a percentage change, the value obtained for an increase of 160 nucleotides would depend on the initial value for the ratio of AMP : UMP. Thus, results are expressed as the increase in the AMP : UMP ratio.

Preparation and sizing of poly(A) tracts. [3H]ATP-containing transcription mixtures containing 10 units of RNasin/ml (Promega Biotec, Madison, Wis., U.S.A.) were incubated at 31 °C for 120 min and transcripts purified as previously described (Hunt, 1983). The RNA was digested with RNase T1 and poly(A) tracts isolated by oligo(dT)-cellulose chromatography (Hunt, 1983). All digestions included [α-32P]UTP-labelled VSV RNA (made in vitro) as an internal control to ensure that RNase T1 digestion was complete. No detectable amount of [32P]UTP-labelled RNA bound to the oligo(dT)-cellulose column after RNase T1 digestion. Poly(A) tracts were electrophoresed on 2% acrylamide-0.5% agarose gels, sliced and counted (Emerson et al., 1977).

Chemicals. [5,6-3H]UTP (24 to 40 Ci/mmole), [2,8-3H]ATP (25 to 50 Ci/mmole), and [α-32P]UTP (> 400 Ci/mmole) were obtained from ICN Pharmaceuticals Chemical and Radioisotope Division. S-Ado-Hcy was obtained from Sigma. P-10 cellulose and DEAE-cellulose were from Whatman and oligo(dT)-cellulose, grade T3, was from Collaborative Research, Lexington, Mass., U.S.A.

RESULTS

Sensitivity of tsG16(I) to S-Ado-Hcy

Rose et al. (1977) reported that addition of 1 mM-S-Ado-Hcy results in an increase in the size of poly(A) tracts made by wt VSV in vitro. We wished to test whether the lesion in tsG16(I) that causes an increase in the size of poly(A) tracts also causes constitutive expression of a property of the transcription complex which in the wt virus is induced by S-Ado-Hcy. If this were so, polyadenylation by tsG16(I) should not be markedly affected by S-Ado-Hcy. Therefore, we measured the ratio of pmol of AMP : UMP incorporated into TCA-insoluble material. The results are shown in Table 1. In the absence of S-Ado-Hcy, the excessive polyadenylation by solubilized virions of tsG16(I) compared to wt VSV was readily detectable by the higher AMP : UMP ratio. Both tsG16(I) and its parental wt virus, wt(Glasgow) showed an increased incorporation of AMP relative to UMP (indicative of increased polyadenylation) in the presence of 1 mM-S-Ado-Hcy. Indeed, the increase in polyadenylation observed upon addition of S-Ado-Hcy was greater for tsG16(I) than for wt(Glasgow).

Magnitude of the polyadenylation response in the presence of S-Ado-Hcy

Rose et al. (1977) did not report the effect of varying the S-Ado-Hcy concentration in their system (which differed from ours in that their system included cell extracts), and so we examined the effect of varying the concentration of S-Ado-Hcy on the AMP : UMP incorporation by

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ratio pmol AMP : UMP incorporated</th>
<th>Increase in AMP : UMP ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt(Glasgow)</td>
<td>1.89</td>
<td>0.47</td>
</tr>
<tr>
<td>tsG16(I)</td>
<td>3.42</td>
<td>1.60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control (no S-Ado-Hcy)</th>
<th>1 mM-S-Ado-Hcy</th>
<th>Increase in AMP : UMP ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt(Glasgow)</td>
<td>1.89</td>
<td>2.36</td>
<td>0.47</td>
</tr>
<tr>
<td>tsG16(I)</td>
<td>3.42</td>
<td>5.02</td>
<td>1.60</td>
</tr>
</tbody>
</table>

* The increase is the difference between the ratio of pmol of AMP : UMP incorporated in the presence of S-Ado-Hcy and the ratio of pmol of AMP : UMP incorporated observed in its absence.
D. M. HUNT, R. MEHTA AND K. L. HUTCHINSON

Fig. 1. The effect of various concentrations of S-Ado-Hcy on polyadenylation by solubilized VSV virions in an in vitro transcription reaction. For each virus, the increase in the AMP : UMP ratio is the difference between the ratio of pmol of AMP : UMP incorporated observed in the presence of a particular concentration of S-Ado-Hcy compared with the ratio observed in the absence of this compound. ●, wt(San Juan); ○, wt(Glasgow); □, tsG16(I).

tsG16(I), wt(Glasgow) and wt(San Juan). The latter is the strain of virus used by Rose et al. (1977) (J. K. Rose, personal communication). The results shown in Fig. 1 indicated that the increased polyadenylation response for each virus had effectively reached a maximum at 100 μM-S-Ado-Hcy. This concentration of S-Ado-Hcy was used in all future experiments. S-Ado-Hcy did not affect the rate of transcription as measured by UTP incorporation (data not shown); this is consistent with the report of Rose et al. (1977) that S-Ado-Hcy does not affect GTP incorporation.

The results in Fig. 1 indicated that all three viruses differed in the magnitude of the polyadenylation response in the presence of S-Ado-Hcy. This observation was repeatable. Upon addition of 100 μM-S-Ado-Hcy, the mean increase in the AMP : UMP ratio was 0.87 (nine experiments, range 0.59 to 1.28) for wt(San Juan), 0.44 (six experiments, range 0.37 to 0.49) for wt(Glasgow) and 1.92 (nine experiments, range 1.34 to 2.70) for tsG16(I). The large difference in response in the presence of 100 μM-S-Ado-Hcy between tsG16(I) and its parental Glasgow wt was unexpected. We wished to determine whether this was because tsG16(I) had acquired a mutation that caused an increase in the magnitude of the response to S-Ado-Hcy, or whether our stocks of wt(Glasgow) had acquired a mutation that suppressed the magnitude of this response. If our stock of wt(Glasgow) had altered over the years, then ts mutants derived in Glasgow at about the same time as tsG16(I) (Pringle, 1970 a, b) should reflect the type of response of tsG16(I) to S-Ado-Hcy rather than that of wt(Glasgow). The increase in the AMP : UMP ratio observed in the presence, compared to the absence, of 100 μM-S-Ado-Hcy was 0.33 for tsG11(I), 0.29 for tsG13(I) and 0.38 for tsG44(IV), suggesting that the original stocks of wt(Glasgow) were comparable to our stock of this virus in their response to S-Ado-Hcy and that tsG16(I) has a mutation which affects the response to this compound.

*Poly(A)* tracts are longer in the presence of S-Ado-Hcy

The AMP : UMP incorporation data do not indicate whether there was an increase in the size of the poly(A) tracts, or merely an increase in number. To check that treatment of tsG16(I) and wt(Glasgow) with S-Ado-Hcy was responsible for the increase in size of poly(A) tracts as
VSV polyadenylation

reported by Rose et al. (1977) for wt(San Juan), poly(A) tracts prepared from RNA made by each virus in the presence or absence of 100 μM-S-Ado-Hcy were subjected to gel electrophoresis (Fig. 2). As reported by Rose et al. (1977), the poly(A) tracts made by wt(San Juan) were much larger when made in the presence of S-Ado-Hcy (Fig. 2d) than in its absence (Fig. 2a); similarly, there was also an increase in size of poly(A) tracts made by wt(Glasgow) (e compared to b) and those made by tsG16(I) (f compared to c).

The L protein modulates the response to S-Ado-Hcy

Since three distinguishable phenotypes for the response to S-Ado-Hcy were observed (Fig. 1), it was possible to determine which virion component was responsible for modulating the response to S-Ado-Hcy. We separated virions into template- and L and NS protein-containing fractions and carried out reconstitution experiments. Since preliminary experiments had shown that the modulation of the response was not a property of the template, all reconstitutions were done with wt(San Juan) template. The effect of 100 μM-S-Ado-Hcy was not detectably altered when template, L protein or NS protein was the rate-limiting component (data not shown). Purified L and NS fractions were assayed for possible contamination with NS or L proteins respectively by assaying each L or NS fraction separately with the template [no incorporation should have been observed since VSV transcription requires L and NS proteins (Emerson & Yu, 1975)].

The results of the reconstitution experiments are shown in Table 2. When the increase in the ratio of AMP : UMP incorporated in the presence, compared to the absence, of 100 μM-S-Ado-Hcy was determined, the results could be separated into three non-overlapping groups according
Table 2. In vitro polyadenylation phenotype in the presence or absence of 100 μM-S-Ado-Hcy observed in transcription reconstitution assays with L and NS fractions derived from wt(San Juan), wt(Glasgow) or tsG16(I) virions, when assayed in various combinations

<table>
<thead>
<tr>
<th>Virion components*</th>
<th>Control (no S-Ado-Hcy)</th>
<th>100 μM-S-Ado-Hcy</th>
<th>Increase in AMP:UMP ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ L + SJ NS</td>
<td>1.74</td>
<td>2.64</td>
<td>0.90</td>
</tr>
<tr>
<td>SJ L + G NS</td>
<td>1.51</td>
<td>2.69</td>
<td>1.18</td>
</tr>
<tr>
<td>SJ L + ts NS</td>
<td>1.62</td>
<td>2.57</td>
<td>0.95</td>
</tr>
<tr>
<td>G L + SJ NS</td>
<td>1.69</td>
<td>2.51</td>
<td>0.82</td>
</tr>
<tr>
<td>G L + G NS</td>
<td>1.58</td>
<td>2.22</td>
<td>0.64</td>
</tr>
<tr>
<td>G L + ts NS</td>
<td>1.76</td>
<td>2.40</td>
<td>0.64</td>
</tr>
<tr>
<td>ts L + SJ NS</td>
<td>3.13</td>
<td>6.67</td>
<td>3.54</td>
</tr>
<tr>
<td>ts L + G NS</td>
<td>3.68</td>
<td>6.49</td>
<td>2.81</td>
</tr>
<tr>
<td>ts L + ts NS</td>
<td>3.38</td>
<td>5.56</td>
<td>2.18</td>
</tr>
</tbody>
</table>

* SJ, wt(San Juan); G, wt(Glasgow); ts, tsG16(I); all reactions contained excess wt(San Juan) template.
† All transcription reconstitution reactions incorporated at least 16 pmol UMP in 120 min at 31 °C. No correction has been made for incorporation attributable to impurities in the fractions used in the reconstitutions. Such incorporation in the template plus L plus NS fractions varied from 0.1 to 1.8 pmol for those reconstitutions containing SJ L protein, from 4.1 to 10.5 pmol for reconstitutions containing G L protein, and from 4.6 to 8.7 pmol for reconstitutions containing ts L protein.
‡ Calculated as in Table 1.

We wished to determine whether the mutation in tsG16(I) which caused an increase in the size of the poly(A) tracts synthesized in vitro was doing so by causing constitutive expression of a property of the transcription complex which is induced in wt VSV upon addition of S-Ado-Hcy (Rose et al., 1977). If this were the case, then addition of S-Ado-Hcy to a tsG16(I) transcription reaction should cause little or no increase in polyadenylation according to whether the mutation was partially or fully constitutive. The results showed that tsG16(I) was fully sensitive to the effects of S-Ado-Hcy on polyadenylation (Table 1, Fig. 1), suggesting that the mutation affecting polyadenylation in tsG16(I) did not mimic the effect of S-Ado-Hcy addition. Indeed, tsG16(I) exhibited a greater increase in polyadenylation in response to saturating levels of S-Ado-Hcy than its parental virus, wt(Glasgow) (see text and Fig. 1). TsG16(I) also showed a greater increase in polyadenylation in response to 100 μM-S-Ado-Hcy than other ts mutants which were derived from the Glasgow strain of VSV (Indiana) at approximately the same time, such as tsG11(I), tsG13(I) and tsG44(IV) (see text). Thus, it seemed that tsG16(I) had a mutation which caused an increase in the polyadenylation response in the presence of S-Ado-Hcy. Since tsG11(I) and tsG13(I), as well as tsG16(I), all belong to complementation group I, which has been assigned to the L gene (Pringle & Szilagyi, 1980; Pringle, 1982), this implies that an abnormally large increase (compared to the parental wt virus) in polyadenylation in response to 100 μM-S-Ado-Hcy is not necessarily linked to thermolability of the L protein.

TsG16(I), wt(Glasgow) and wt(San Juan) all clearly differed in the extent of the increase in polyadenylation observed in the presence of saturating amounts of S-Ado-Hcy (see text and Fig. 1). It was possible, therefore, to do transcription reconstitution experiments using purified template and L and NS fractions from the various viruses to see which, if any, of these subviral
fractions modulated the polyadenylation response to S-Ado-Hcy. The results (Table 2) indicated that it was the L protein of each virus which modulated the polyadenylation response to S-Ado-Hcy.

We have not been able to select non-ts revertants of tsG16(I) and so do not know if the L protein mutation which causes temperature sensitivity (Hunt et al., 1976) is the same as that which causes aberrant polyadenylation (Hunt et al., 1984) and/or the same as the L protein mutation which caused an altered polyadenylation response to S-Ado-Hcy. As yet, there is no conclusive evidence to indicate which protein is the poly(A) polymerase. However, the results reported here provide further circumstantial evidence that the L protein is at least intimately involved in controlling poly(A) synthesis.

We thank Edith Smith and Ramon Cook for technical assistance. We also thank P.-K. Chang, who participated in some of the preliminary experiments. This work was supported by Public Health Service grants AI 18201 and GM 36820.

REFERENCES


(Received 1 March 1988)