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Host range mutants of vesicular stomatitis virus defective in *in vitro* RNA methylation

(*in vitro* transcription/capping)

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ABSTRACT **The viral RNA polymerase of detergent-treated vesicular stomatitis virus normally synthesizes viral mRNAs *in vitro* that are both guanylylated and methylated to give 5′-terminal 7mGpppA^m caps. We have characterized a virus host range mutant, *hr* 1, that is totally defective *in vitro* in the methylation of mRNA, although full-length polyadenylylated mRNAs with 5′ termini of the form GpppA are synthesized in normal yields. A second mutant, *hr* 8, is partially defective in methylation and synthesizes mRNAs *in vitro* with primarily GpppA and some GpppA^m 5′ termini. When used for *in vitro* translation, the unmethylated *hr* 1 mutant mRNA shows, as expected, reduced synthesis of viral proteins. These data provide direct evidence that the vesicular stomatitis virus-associated methyltransferase activities are virus encoded.**

Vesicular stomatitis virus (VSV) has a negative-sense single-stranded RNA genome. Using the virion-associated RNA polymerase (1), the virus catalyzes the *in vitro* synthesis of all five viral mRNAs (2). The enzymatic machinery required for transcription is complex, because the mRNA products are modified, first by guanylylation and methylation to yield 5′ termini with caps of the form 7mGpppA^{m}pAp (3) and second by polyadenylylation at the 3′ termini (4, 5). In the absence of the methyl donor, *S*-adenosylmethionine (AdoMet), total *in vitro* RNA synthesis is virtually unaffected and the mRNAs contain the guanylylated, but unmethylated, cap GpppApAp (6). Studies by Emerson and co-workers (7, 8) have shown that all of the components of the VSV nucleocapsid, the genome RNA–N protein complex as well as the proteins L and NS, are essential for *in vitro* transcription. None of the requisite enzymatic activities—i.e., those for polymerization, guanylylation, methylation, or polyadenylylation—have, however, been assigned to any specific viral protein. Indeed, it has not yet been established whether the capping and methylating activities are of viral or host origin.

Because of our interest in the role of the host cell in VSV reproduction, we have studied a series of VSV host range (*hr*) mutants that were originally isolated by Simpson and Obijeski (9, 10). These mutants grow well at 35°C in permissive baby hamster kidney cells or chicken embryo fibroblasts but are severely restricted in many human cell lines, including HEp-2 cells (11). Studies by Simpson and co-workers (10, 12) of the host range defect have shown that the mutants exhibit an overall RNA$^-$ phenotype in infected nonpermissive cells at 35°C. Most of these mutants are also thermosensitive at higher temperatures (39°C) in the permissive host. Whether the thermolability expressed at the higher temperature is related to the host range phenotype at lower temperatures is not known. We show here that two of the VSV host range mutants, *hr* 1 and *hr* 8, are defective in mRNA methylation *in vitro*, although RNA synthesis, guanylylation, and polyadenylylation are unaffected. These data provide direct evidence that the VSV-associated methyltransferase activities are virus encoded.

MATERIALS AND METHODS

Growth and Purification of Virus. Wild-type (WT) VSV (Indiana serotype), *hr* 1, and *hr* 8 (supplied by R. W. Simpson, Rutgers University) were grown on monolayer cultures of permissive baby hamster kidney (BHK-21) cells. To prepare virus for transcription experiments, BHK cells were infected with either the mutant or WT VSV (the parental virus) at a multiplicity of 0.1 plaque-forming units per cell and incubated for 24 hr at 35°C or 37°C, respectively. WT VSV grown at either 37°C or 35°C gave identical *in vitro* methylation patterns. The released virus was purified as described (5). The purified virus was suspended at 2–4 mg/ml in 1 mM Tris·HCl, pH 7.4/1 mM EDTA/10% $(CH_3)_2SO$ and stored at −70°C.

***In Vitro* Synthesis and Purification of RNA.** RNA was synthesized *in vitro* from detergent-activated purified virus in a standard incubation mixture as described (2); the amount of each virus preparation giving maximal transcription was determined experimentally. Incubation was at 30°C for 5 hr. When [α-^{32}P]ATP (Amersham; 410 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was used as a substrate, the concentration of ATP was reduced to 500 μM. For methylation experiments, RNA was synthesized in the presence of [^{3}H]AdoMet (Amersham; 65 Ci/mmol) as indicated in the text or [^{3}H]UTP (New England Nuclear; 40.4 Ci/mmol) at 25 μCi/ml. Total incorporation of radioactivity into RNA was determined according to the procedure of Pugh *et al.* (13).

For the isolation of RNA, the reaction mixture was treated with proteinase K (250 μg/ml) in 50 mM NaOAc, pH 5.1/100 mM NaCl/10 mM EDTA/0.5% $NaDodSO_4$ containing sodium heparin at 50 units/ml for 30 min at 37°C. The RNA was extracted with phenol/chloroform and separated from the substrates by Sephadex G-25 chromatography. Poly(A)$^+$RNA was isolated by binding to and elution from oligo(dT)-cellulose as described (4).

RNA Sizing and Cap Analysis. Purified [^{3}H]UTP-labeled poly(A)$^+$RNA was analyzed by electrophoresis on 1.5% agarose/6 M urea gels as described (14). The gels were fixed in acetic acid, processed for fluorography (15), dried, and exposed to Kodak X-OMat film at −70°C.

The 5′ termini of the [^{32}P]ATP- and [^{3}H]AdoMet-labeled RNAs were identified by digesting the RNA with specific RNases and then using high-voltage paper electrophoresis or chromatographic procedures as described (16). Briefly, the mRNAs were digested with RNase T2 (75 units/ml), RNase A (100 μg/ml), and RNase T1 (20 units/ml) in 20 mM NaOAc (pH 5.0) for 24 hr at 37°C. The terminal oligonucleotides were

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Abbreviations: VSV, vesicular stomatitis virus; AdoMet, *S*-adenosylmethionine; WT, wild type; AdoHcy, *S*-adenosylhomocysteine.

isolated and digested with nuclease P1 (200 μg/ml) in 20 mM NaOAc (pH 6.0) for 1 hr at 37°C.

Isolation of RNA for *in Vitro* Translation. RNA was synthesized in the standard reaction mixture as described above using 500 μg of WT or *hr* 1 VSV, alone or in the presence of 20 μM AdoMet or 100 μM S-adenosylhomocysteine (AdoHcy). The mRNA was isolated by the procedure of Cooper and Moss (17), as modified by G. D. Brown and R. W. Moyer (personal communication). Briefly, the reaction mixture was adjusted to 2% Sarkosyl/5 mM EDTA and then centrifuged at 30,000 rpm for 1 hr at 4°C in a Beckman Ti 50 rotor. CsCl was added to the supernatant to 0.8 mg/ml, the mixture was layered on a 5.7 M CsCl cushion, and the RNA was pelleted in the SW 41 rotor at 30,000 rpm for 16 hr at 20°C. RNA was precipitated several times with EtOH to remove any residual CsCl and stored under EtOH at −20°C.

***In Vitro* Translation of VSV RNA.** The *in vitro*-synthesized RNA was pelleted from EtOH and suspended in water at 1 mg/ml. The RNA was translated in a mRNA-dependent rabbit reticulocyte lysate prepared and optimized as described in refs. 17–19. The translation reactions (25 μl) were optimal with RNA at 160 μg/ml and were carried out at 37°C for 30 min in the presence of [^{3}H]leucine (1.3 mCi/ml; 45 Ci/mmol; New England Nuclear). The translation products were diluted 1:10 with RIPA buffer (20) and immunoprecipitated with anti-VSV serum by the procedure of Kessler (21). The antibody-precipitated proteins were analyzed by 10% polyacrylamide/NaDodSO$_4$ gel electrophoresis as described (22) followed by fluorography (15). The VSV protein bands were cut from the gel and solubilized with 30% H_2O_2 at 60°C, and the samples were assayed in scintillation fluid.

RESULTS

***In Vitro* RNA Synthesis and Methylation.** WT VSV and the mutant viruses were purified and used for *in vitro* transcription in an AdoMet-lacking reaction mixture with [^{3}H]UTP as the labeled substrate. At the permissive temperature, the level of *in vitro* RNA synthesis by *hr* 1 was comparable with that of WT VSV (Table 1), confirming the data of Simpson and Obijeski (9), while *in vitro* RNA synthesis by *hr* 8 was somewhat reduced. The RNA was isolated and fractionated by chromatography on oligo(dT)-cellulose. For each of the three viruses, 70–80% of the RNA product bound to the column and thus contained poly(A) sequences (data not shown). Analysis of the poly(A)$^+$RNA by acid/urea agarose gel electrophoresis (Fig. 1) showed that WT VSV and *hr* 1 synthesized identical G, N, NS, and M mRNAs. By quantitation of the radioactivity in the gel bands, the ratios of the *in vitro* mRNAs for *hr* 1 (N, 1.0; G, 0.6; M/NS, 0.8) were very similar to those of the WT VSV (N, 1.0; G, 0.8; M/NS, 0.7). Similar results were obtained for *hr* 8 *in vitro* RNAs (data not shown). Each *in vitro* mRNA, however, has an electrophoretic mobility that is less than that of the species synthesized *in vivo*. The analyses of [^{3}H]ATP-labeled WT VSV and *hr* 1 products have shown that all these *in vitro* RNAs have poly(A) tails about 100 bases longer than those of mRNAs from WT VSV-infected cells (data not shown).

Table 1. *In vitro* VSV RNA synthesis and methylation

Virus	AdoHcy	^{3}H incorporated, cpm	
		RNA synthesis	Methylation
WT VSV	−	293,390	22,604
WT VSV	+	365,922	765
hr 1	−	224,733	640
hr 1	+	204,549	566
hr 8	−	124,438	2,384
hr 8	+	181,679	782
None	−	310	570

VSV RNA was synthesized *in vitro* in the presence (+) or absence (−) of 100 μM AdoHcy. Synthesis and methylation were measured as incorporation of [^{3}H]UTP (25 μCi/ml) and [^{3}H]AdoMet (0.154 μM; 10 μCi/ml), respectively, during *in vitro* VSV transcription with purified WT VSV, *hr* 1, and *hr* 8.

The purified virus preparations were also tested for their ability to catalyze RNA methylation during *in vitro* transcription by using [^{3}H]AdoMet (0.154 μM) as the labeled substrate. The data (Table 1) show that, although WT VSV yields significant *in vitro* methylation of the RNA product, the RNA synthesized by *hr* 1 is not detectably methylated. In addition, although the presence of the methylation inhibitor AdoHcy has some effect (20–25%) on RNA synthesis (positive and negative, depending on the virus), the methylation activity of WT VSV is abolished, while that of *hr* 1 is unaffected, as expected. In contrast to *hr* 1, mutant *hr* 8 does show a small amount of AdoHcy-sensitive methylation of the *in vitro* RNA product. Identical methylation patterns are obtained for each virus when the AdoMet concentration in the *in vitro* reaction is increased to 2 μM (see Figs. 2 and 5). Mutants *hr* 1 and *hr* 8, therefore, have defects in RNA methylation that do not affect overall RNA synthesis or polyadenylylation.

Identification of the 5′ Termini of WT VSV and *hr* 1 *in Vitro* RNAs. To analyze the structure of the 5′ termini of the methyl-deficient *hr* 1 RNA compared with WT VSV RNA, product was synthesized *in vitro* in the presence of both [α-^{32}P]ATP and [^{3}H]AdoMet (2 μM). Poly(A)$^+$RNA was isolated and digested with RNases A, T1, and T2 to yield 3′-ribonucleoside monophosphates and any additional species derived from the 5′ termini of the molecules. DEAE-cellulose paper electrophoresis of the products from WT VSV shows two peaks (I and II) that contain both ^{32}P and [^{3}H]methyl labels (Fig. 2*A*). Based on our previous studies (13) and those of Testa and Banerjee (23), we predict that the slower migrating WT VSV peak I represents the monomethylated terminus, GpppA^{m}pAp, and the faster migrating peak II represents the fully methylated terminus, 7mGpppA^{m}pAp. In contrast, mutant *hr* 1 RNA has a single nonmethylated terminal oligonucleotide (peak III, Fig. 2*B*) that has a mobility slightly faster than that of peak I from WT VSV (Fig. 2*A*).

To identify the 5′ termini, peaks I, II, and III (Fig. 2*A*) were separately eluted and digested with nuclease P1, which removes 3′-phosphate groups and releases 5′ mononucleotides but leaves the cap structure intact. The products were analyzed by electrophoresis on 3MM paper (Fig. 3). Nuclease P1 diges-

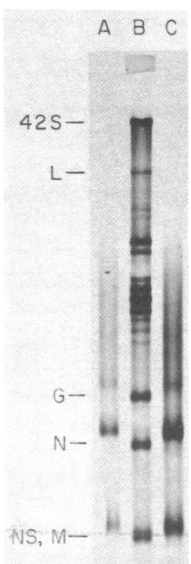

FIG. 1. Acid/urea/agarose gel electrophoresis of mutant *hr* 1 and WT VSV mRNAs transcribed *in vitro*. VSV RNAs were synthesized *in vitro* with [^{3}H]UTP at 100 μCi/ml and the poly(A)$^+$mRNAs were isolated and analyzed by acid/urea/agarose gel electrophoresis. Lanes: A and C, poly(A)$^+$RNA products from purified WT VSV and *hr* 1, respectively; B, total [^{3}H]uridine-labeled RNAs synthesized *in vivo* in WT VSV-infected BHK cells. Markers were the VSV 42S genome RNA and the mRNAs for the L, G, N, NS, and M proteins.

tion of WT VSV peak I (GpppA^{m}pAp) (Fig. 2A) yields pA and a methylated cap (637 and 598 ^{32}P cpm, respectively) that migrates in the position in which both GpppA and GpppA^m migrate (Fig. 3A). For the WT VSV oligonucleotide in peak II (Fig. 2A), nuclease P1 digestion yields approximately equal ^{32}P radioactivity in pA and the cap 7mGpppA^m (633 and 588 cpm, respectively; Fig. 3B), consistent with its predicted identity of 7mGpppA^{m}pAp. Cap 7mGpppA^m from peak II and cap GpppA^m from peak I are present in equal amounts, with [^{3}H]-methyl/^{32}P cpm ratios of 7,750:588 and 3,837:598, respectively (Fig. 3 A and B), which are consistent with their identification as the dimethyl and monomethyl caps. Further analysis of these caps by chromatography in a solvent system that separates methylated oligonucleotides on the basis of their base sequence and the number of methyl substituents (24) confirmed that the caps from WT VSV peaks I and II (Fig. 2A) are GpppA^m (Fig. 4A) and 7mGpppA^m (Fig. 4B), respectively.

Nuclease P1 digestion of the *hr* 1 oligonucleotide in peak III (Fig. 2B) yielded predominantly (75–80%) a nonmethylated cap migrating similar to GpppA and GpppA^m (Fig. 3C). This species was conclusively identified as GpppA by its comigration with authentic marker during chromatography (Fig. 4C), and thus peak III (Fig. 2B) must have been GpppAp. Further digestion of the [α-^{32}P]ATP-labeled cap with nucleotide pyrophosphatase yielded ppA and pA (data not shown), which are the expected reaction products for the GpppA cap.

In other experiments (data not shown), we have examined the 5′ termini of WT VSV and *hr* 1 RNA products synthesized under other conditions. As shown above, RNA synthesized by WT VSV in the presence of 2 μM AdoMet contains equal amounts of the caps GpppA^m and 7mGpppA^m and some pppA

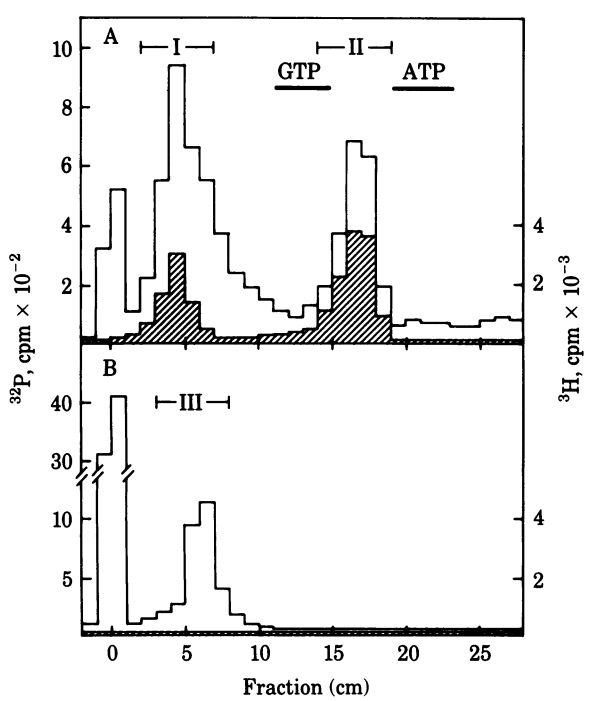

FIG. 2. High-voltage electrophoresis analysis of the 5′-terminal oligonucleotides of WT VSV (A) and *hr* 1 (B) mRNAs synthesized *in vitro*. mRNAs were synthesized *in vitro* in the presence of both [α-^{32}P]ATP (open bars) at 400 μCi/ml and [^{3}H]AdoMet (hatched bars) at 180 μCi/ml (2 μM). The poly(A)$^+$RNAs were isolated and purified and the RNA was digested with RNases T2, T1, and A and analyzed by electrophoresis on DEAE-cellulose paper in pyridine/acetate buffer, pH 3.5, at 1,750 V for 16 hr. Appropriate marker compounds were co-electrophoresed with the samples and located by UV absorption. Material at the origin is incompletely digested RNA and represents a small portion that, in multiple experiments for each virus, varied from 0.04% to 0.4% of the total product digested.

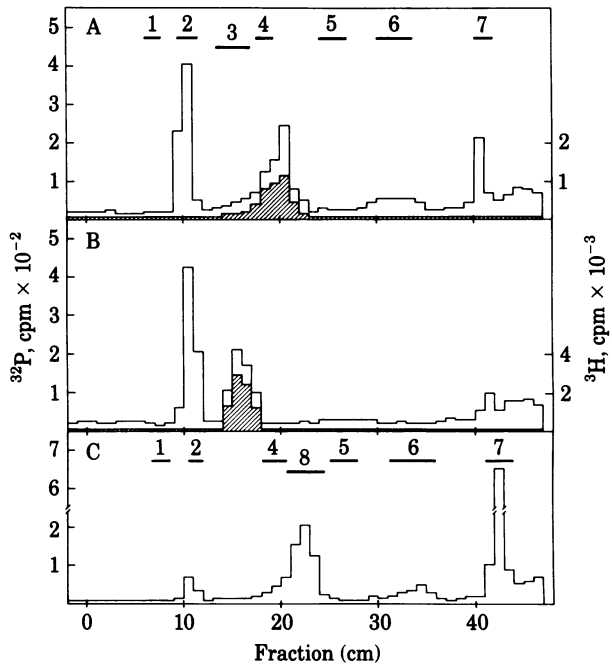

FIG. 3. High-voltage electrophoresis analysis of nuclease P1-digested 5′-terminal oligonucleotides from WT VSV and *hr* 1 mRNAs synthesized *in vitro*. The 5′-terminal oligonucleotides (peaks I and II, Fig. 2A, and peak III, Fig. 2B) from the WT VSV and *hr* 1 RNAs, respectively, were individually eluted and digested with nuclease P1, and the products were analyzed by electrophoresis on Whatman 3MM paper in pyridine/acetate buffer, pH 3.5, at 3,000 V for 2 hr. Products were labeled with [α-^{32}P]ATP (open bars) and [^{3}H]AdoMet. (A) WT VSV peak I. (B) WT VSV peak II. (C) *hr* 1 peak III. Markers: 1, pC; 2, pA; 3, 7mGpppA^m; 4, pG; 5, pU; 6, pppA; 7, P$_i$; 8, GpppA. Those in A and B were identical.

ends (15%). However, at 20 μM AdoMet, the fully methylated cap 7mGpppA^m is the predominate product. In the absence of AdoMet or in the presence of added AdoHcy, WT VSV RNA products contain mostly GpppA (75–80%) and some pppA ends just as does *hr* 1 RNA (Fig. 3C). In the case of *hr* 1, only GpppA and some pppA termini (15–20%) are found under each of the above conditions, including that at the highest AdoMet concentration (20 μM).

Similar 5′-termini analyses were carried out on the WT VSV and *hr* 1 poly(A)$^-$RNAs with sedimentation values of 2–5 S as determined by gradient centrifugation. This size class would include the small RNA products typical of *in vitro* transcription reactions, predominantly the leader RNA (25), as well as the small internally initiated RNAs described by Testa *et al.* (26). The relative amounts of synthesis of these small poly(A)$^-$RNAs were the same for WT VSV and *hr* 1 and they contained only pppA 5′ termini (data not shown), as expected for the leader RNA.

Identification of the 5′ Termini of the mRNAs Synthesized *in Vitro* by *hr* 8. To determine the structure of the 5′ termini of the partially methylated mRNA synthesized by *hr* 8 (Table 1), product was labeled *in vitro* with both [α-^{32}P]ATP and [^{3}H]AdoMet. Poly(A)$^+$RNA was digested with RNases A, T1, and T2 and analyzed by DEAE-cellulose paper electrophoresis (Fig. 5A). In contrast to the single unmethylated 5′ oligonucleotide observed with *hr* 1 (Fig. 2A), *hr* 8 product contains three terminal oligonucleotides. From the analyses described above of WT VSV and *hr* 1 products, we predict that *hr* 8 peak I contains a mixture of two oligonucleotides, GpppAp and GpppA^{m}pAp, and peak II represents the fully methylated oligonucleotide, 7mGpppA^{m}pAp. The materials in peaks I and II (Fig. 5A) were separately eluted and digested with nuclease P1,

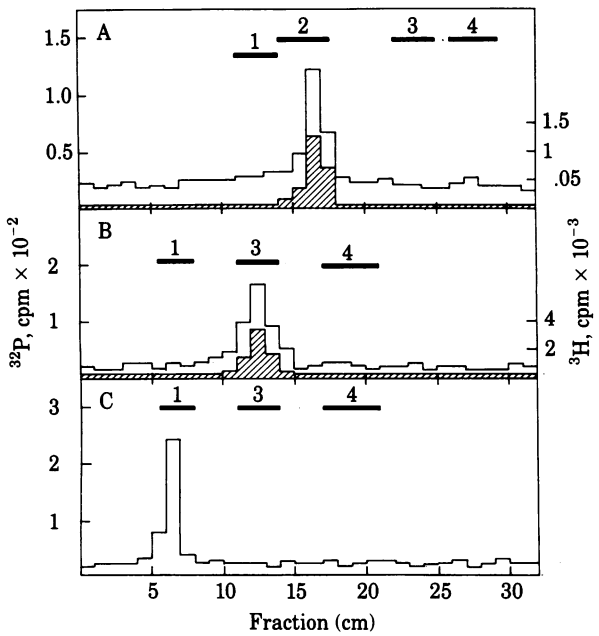

FIG. 4. Chromatographic analysis of WT VSV and *hr* 1 5′-terminal caps. The three nuclease P1-digested VSV caps (Fig. 3 *A–C*) were eluted separately and analyzed directly by descending chromatography on Whatman 3MM paper with isobutyric acid/0.5 M ammonium hydroxide, 10:6 (vol/vol) as the solvent. The 5′-terminal caps were labeled with [α-^{32}P]ATP (open bars) and [^{3}H]AdoMet (hatched bars). (*A*) WT VSV cap from Fig. 3*A*. (*B*) WT VSV cap from Fig. 3*B*. (*C*) Mutant *hr* 1 cap from Fig. 3*C*. Markers: 1, GpppA; 2, GpppA^{m}; 3, 7mGpppA^{m}; 4, pA.

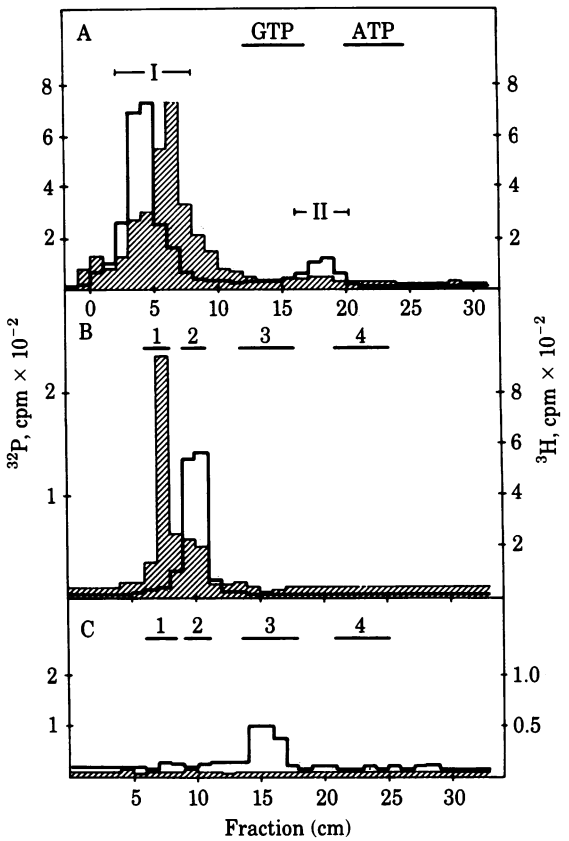

FIG. 5. High-voltage electrophoresis analysis of the 5′-terminal oligonucleotides of *hr* 8 mRNAs synthesized *in vitro*. RNAs were synthesized *in vitro* by *hr* 8 in the presence of both [α-^{32}P]ATP (hatched bars) at 400 μCi/ml and [^{3}H]AdoMet (open bars) at 180 μCi/ml (2 μM). The poly(A)$^{+}$RNA was isolated, digested with RNases T2, T1, and A and analyzed by electrophoresis on DEAE-cellulose paper (*A*). Peaks I and II were eluted and digested with nuclease P1, and the caps were purified by electrophoresis on Whatman 3MM (data not shown). (*B* and *C*) Purified caps from *hr* 8 peaks I and II, respectively, were eluted and analyzed by chromatography. Markers: 1, GpppA; 2, GpppA^{m}; 3, 7mGpppA^{m}; 4, pA.

and the 5′-terminal caps from each peak were purified by electrophoresis on 3MM paper as in Fig. 3 (data not shown). The caps were then eluted and analyzed by chromatography. The caps from *hr* 8 peak I (Fig. 5*A*) are identified as GpppA and GpppA^{m} (Fig. 5*B*), while the cap from peak II is 7mGpppA^{m} (Fig. 5*C*). From the ratio of [^{32}P]ATP incorporated into each species, the GpppA/GpppA^{m}/7mGpppA^{m} ratio is 7:2:0.2 in *hr* 8 RNAs synthesized *in vitro* in the presence of 2 μM AdoMet. In summary, we have identified two VSV mutants that are deficient in methylation but behave normally in all other aspects of *in vitro* transcription, including proper initiation and synthesis of the small poly(A)$^{-}$RNAs and the formation of normal-sized mRNA products that are guanylylated and polyadenylylated.

***In Vitro* Translation of VSV Transcription Products.** The experiments described above indicate that *hr* 1 synthesizes mRNAs *in vitro* that are normal except for the absence of methyl groups. To confirm that accurate transcription occurs, we have used *in vitro* translation assays to show that the *hr* 1 *in vitro* mRNAs are indeed functional. As controls, RNA product was synthesized with WT VSV in the presence of AdoMet to give fully methylated caps (7mGpppA^{m}) or of AdoHcy, where most of the RNA has GpppA caps. Similarly, RNA was synthesized by *hr* 1 alone or in the presence of the same agents, where, in each case, the product contained largely GpppA caps (Fig. 3*C*).

Constant amounts of *in vitro* RNA were translated in rabbit reticulocyte lysates and VSV-specific proteins were separated by polyacrylamide gel electrophoresis. Both WT and mutant RNAs direct the synthesis of the NS, N, and M proteins with mobilities identical to those of VSV marker proteins, showing that the *in vitro* RNA products yield viral proteins of normal size. We detected little, if any, *in vitro* synthesis of the nonglycosylated G protein or of the L protein. Moreover, the amounts of the viral proteins synthesized depended on the nature of the 5′ termini of the mRNA (Table 2). When the fully methylated RNA (WT VSV/20 μM AdoMet) was used, high levels of translation occurred, yielding all three viral proteins, whereas the WT VSV RNA containing GpppA (WT VSV/AdoHcy) ends gave a much reduced level of translation, 15% and 25% of the N and M proteins, respectively. Synthesis of the NS protein, for unknown reasons, seems to be affected to a lesser degree by the absence of the methyl groups. The RNA transcribed from *hr* 1 under all of our conditions yielded levels of translation of each protein similar to that of the nonmethylated WT VSV RNA, as expected because these RNAs also contain mostly GpppA caps. A similar dependence of translation efficiency in rabbit reticulocyte lysates on the presence of RNAs with methylated caps was described earlier by Lodish and Rose (27). Our data support the results of our structural analyses showing that the *hr* 1 RNA is unmethylated.

DISCUSSION

Capping and methylation of the mRNA products transcribed *in vitro* occurs for a number of animal viruses that contain virion-associated RNA polymerase activities, including VSV, reovirus, vaccinia virus, and Newcastle disease virus (for review, see ref. 28). Only in the case of vaccinia virus have the relevant virion-associated enzymes, guanylyltransferase, guanine-7-methyltransferase, and a nucleoside 2′-methyltransferase, actually been isolated and characterized (29). The vaccinia enzymes appear to be virus encoded, since they are associated with the

Table 2. Analysis of the *in vitro* translation products of VSV *in vitro* transcripts

RNA	AdoMet, μM	Protein, cpm NS	N	M
WT VSV	20	19,096	39,214	6,307
WT VSV	100	9,241	5,767	1,558
hr 1	—	6,282	7,754	1,571
hr 1	20	3,215	6,891	885
hr 1	100	2,777	3,701	760

RNA product was synthesized from WT VSV or *hr* 1 and purified, and a constant amount (4 μg) of each RNA was translated in a rabbit reticulocyte lysate. The translation products were immunoprecipitated and separated by polyacrylamide gel electrophoresis, and the individual protein bands were cut from the gel and assayed.

virion and appear only in vaccinia virus-infected cells (30); however, no viral mutants in these enzymes have been described. We have characterized two VSV host range mutants isolated by Simpson and Obijeski (9) that are defective specifically in mRNA methylation *in vitro*, and we conclude, therefore, that this activity is virus encoded.

On the basis of their *in vitro* transcription and methylation studies, Testa and Banerjee (23) proposed that the two terminal methylations on VSV mRNA were catalyzed by two different methyltransferase activities that act sequentially in the order GpppA → GpppA^m → 7mGpppA^m. However, our data from analysis of the VSV mRNAs synthesized *in vivo* in the presence of the methylation inhibitor cycloleucine (16) suggest that the reverse order (GpppA → 7mGpppA → 7mGpppA^m) can also occur, at least within the infected cell. The methylation defective mutant, *hr* 1, described here methylates neither the guanosine nor the penultimate adenosine of the cap (Fig. 2). We cannot distinguish in this case whether both methylating activities are deficient or whether a single defect in the first event *in vitro*—i.e., the 2′-*O*-adenosine methylation—may also preclude the subsequent guanosine methylation in the ordered process observed *in vitro*. We also show here that another of the VSV host range mutants, *hr* 8, can partially methylate RNA synthesized *in vitro* (Fig. 5), primarily at the adenosine nucleotide of the cap. These experiments show that these two mutants have different methylation capacities. They further suggest that, while *hr* 1 is clearly lacking 2′-*O*-methyladenosine transferase activity, *hr* 8, although it has the partial capacity to methylate adenosine, appears to be unable to catalyze the methylation of guanosine.

In their earlier characterization of the *hr* mutants, Simpson *et al.* (11) showed that low levels of complementation of the host range defect occurred between some of the mutants (including *hr* 1 and *hr* 8) in mixed infections. On the basis of these data, they postulated that the *hr* defects resided in the large VSV L protein. Szilagyi *et al.* (31) have, in fact, identified by reconstitution experiments the L protein as being defective in a temperature-dependent host range mutant, although the nature of the defect in this mutant was not identified. Similar reconstruction experiments with the *hr* 1 and 8 mutants should conclusively identify the viral protein(s) responsible for methylation.

The host range mutants described here can be propagated on several permissive cell lines, including chicken embryo fibroblasts and BHK cells. Simpson *et al.* (11) have shown that a number of human cell lines are nonpermissive to various degrees for the growth of *hr* 1. In our characterization of the nonpermissive *hr* 1 and *hr* 8 infections of HEp-2 cells, we have shown that primary transcription does occur, as did Obijeski and Simpson (10). However, we find little, if any, viral protein synthesis and no replication of the genome RNA (ref. 32; unpublished data). The discovery of a RNA methylation defect associated with a host range phenotype was unexpected. The interesting question is, therefore, how the permissive cell corrects this viral defect. Two hypotheses seem possible. First, the permissive cells may have a higher cytoplasmic level of the alternative host methyltransferase enzymes, such that the undermethylated VSV mRNAs become properly modified. Second, the defective viral enzyme, perhaps as is the case for *hr* 8, may have an unusually high K_m for the AdoMet substrate, which is compensated for by high intracellular AdoMet concentrations in permissive cells. In this regard, we have previously shown (32) that coinfection with rabbit poxvirus can rescue both *hr* 1 and *hr* 8 from their nonpermissive infections of HEp-2 cells. We suggest that this is accomplished via the first mechanism—i.e., by synthesis of the cytoplasmic poxvirus-specific methyltransferase enzymes that properly modify the VSV mRNAs to allow for normal translation, which then allows VSV replication.

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