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Transcriptional Activity and Mutational Analysis of Recombinant Vesicular Stomatitis Virus RNA Polymerase

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The 241-kDa large (L) protein of vesicular stomatitis virus (VSV) is the multifunctional catalytic component of the viral RNA polymerase. A protocol has been developed for the synthesis of recombinant L protein that will support viral mRNA synthesis in vitro. COS cells were transfected with a transient expression vector (pSV-VSL1 [M. Schubert, G. G. Harmison, C. D. Richardson, and E. Meier, Proc. Natl. Acad. Sci. USA 82:7984–7988, 1985]) which contains the simian virus 40 late promoter for the transcription of a cDNA copy of the L protein of the Indiana serotype of VSV. Cytoplasmic extracts of these cells efficiently transcribed VSV mRNAs in vitro in conjunction with N protein-RNA template purified from virus and recombinant phosphoprotein synthesized in *Escherichia coli*. mRNA synthesis was completely dependent upon addition of both bacterial phosphoprotein and extracts from cells transfected with the L gene. Extracts from mock-transfected cells or from cells transfected with the expression vector alone did not support VSV RNA synthesis. RNA synthesis was proportional to the concentration of cell extract used, with an optimum of 0.2 mg/ml. Rhabdoviruses and paramyxoviruses contain a highly conserved GDNQ motif which was mutated in the transfected L gene. All constructs with mutations within the core GDN abrogated transcriptional activity except for the mutant containing GDD, which retained 25% activity. Conserved amino acid changes outside of the core GDN and changes corresponding to other paromyxovirus and rhabdovirus L proteins retained variable transcriptional activity. These findings provide experimental evidence that the GDN of negative-strand, nonsegmented RNA viruses is a variant of the GDD motif of plus-strand RNA viruses and of the XDD motif of DNA viruses and reverse transcriptases.

The genome RNA of positive-strand RNA viruses can immediately be translated upon uncoating within a cell, allowing synthesis of the viral transcriptional machinery required for subsequent mRNA synthesis and replication. This is not the case with negative-strand RNA viruses, as the genomic RNA is not mRNA sense. As a result, these viruses must contain all the viral (and host?) proteins required for primary mRNA synthesis within the infected cell. In the case of vesicular stomatitis virus (VSV), detergent-disrupted virions can direct mRNA synthesis in vitro efficiently, making it a model virus for the study of transcription and its regulation in vitro for many years (1, 2).

Only three of the virus-encoded proteins within the VSV particle are required for RNA synthesis (11). The nucleocapsid (N) protein encapsidates the negative-strand genome RNA, forming the N-RNA complex, which is the only form in which the RNA is recognized by the other components of the viral transcriptional machinery. The RNA polymerase of VSV contains two viral proteins (11): the phosphoprotein (P protein), which is a noncatalytic transcription factor known also as the nonstructural (NS) protein; and a large (L) protein, which is the presumptive RNA-dependent RNA polymerase. The N-RNA complex, the P protein, and the L protein can each be separately isolated from purified virions and added back to each other to reconstitute mRNA synthesis (8, 11).

The best studied of these three viral proteins at the molecular level is the P protein, primarily because recombinant P protein can be translated in vitro (15) or in *Escherichia coli* (3), which will support transcription of the N-RNA template in association with L protein purified from virions. Molecular analysis of recombinant VSV N protein is also currently under way (7a).

The least understood, and perhaps the most interesting, component of the VSV RNA polymerase is the L protein. The L protein does not function as an RNA-dependent RNA polymerase unless associated with the P protein. The L protein appears to be multifunctional because many of the catalytic requirements of VSV mRNA synthesis have been assigned to the L protein; these requirements include RNA initiation and elongation (10), polyadenylation (19), guanylylation and methylation of nascent transcripts (18), and phosphorylation of the P protein to a transcriptionally active state (4). However, most of these properties have been demonstrated only indirectly by biochemical or complementation studies, and none have been assigned to specific regions of the L protein. Thus, the precise function of the multifunctional L protein of VSV is not as well understood as the functions P and N proteins because of its extreme size: at 241 kDa, it cannot be expressed as a recombinant molecule either in vitro from mRNAs transcribed from recombinant DNA or in *E. coli* (2a). Thus, manipulation of the L protein at the amino acid level to examine the individual catalytic processes of VSV transcription has not proven possible. Moreover, while there are conserved blocks of amino acid homology, there is relatively little overall amino acid sequence conservation between the L proteins of different viruses, complicating comparative studies (5, 28).

However, recombinant L protein has been expressed in vivo in transfected COS cells (31) from a cDNA copy of the L gene inserted into a eukaryotic transient expression vector (34) under control of the late promoter of simian virus 40 (SV40). The expressed L protein was detectable by immu-

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noprecipitation and was biologically active by virtue of its ability to complement L-deficient VSV mutants (31).

Using this SV40-based transient expression system, we have also transfected COS cells to produce biologically functional L protein. Initially, our aim was to purify the recombinant L protein synthesized in the transfected cells to reconstitute VSV transcription in vitro. However, we developed a simpler protocol using crude cytoplasmic extracts (33) from these transfected cells that will efficiently support VSV transcription in vitro in conjunction with N-RNA template purified from virions and recombinant P protein expressed in bacteria (3). This system has allowed, for the first time, mutational analyses of the RNA polymerase of a negative-strand, nonsegmented RNA virus. Finally, in this report, we provide evidence that a conserved GDN motif is a variant of the GDD motif found in many other RNA-dependent RNA polymerases and the XDD motif of DNA-dependent RNA polymerases (23).

MATERIALS AND METHODS

Plasmids. pSV-VSL1 (31), which contains the L gene of the Indiana serotype of VSV [VSV(I)] under the transcriptional control of the SV40 late promoter in the transient expression vector pJC119, was generously provided by Manfred Schubert. pJC119 (34) was reconstructed from pSV-VSL1 by excision of the L cDNA by digestion with *Xho*I and religation. pET-3a-P(I) (3), which expressed VSV(I) P protein [P(I)] in *E. coli*, was provided by Sailen Barik.

Site-directed mutagenesis of the L gene. The conserved GDNQ motif of the L genes of rhabdoviruses and paramyxoviruses (28) is directly adjacent to a unique *Dra*III site within pSV-VSL1. Digestion of pSV-VSL1 at this *Dra*III site, which cuts at nucleotide 2137 of the L(I) gene, and a unique *Sac*I site (which cuts at nucleotide 2440) removed a 303-nucleotide fragment which was replaced by polymerase chain reaction (PCR)-amplified products containing the respective mutations. Oligonucleotides containing the mutations in the GDNQ motif therefore spanned the *Dra*III site and the GDNQ motif and extended for a further 20 nucleotides. The upstream oligonucleotide for PCR was complementary to nucleotides 2447 to 2771 of the L gene, resulting in PCR products which contained the unique *Sac*I site at 2436 (5). Mutant L sequences were identified by sequencing the amplified 220-nucleotide region in its entirety, ensuring that additional mutations were not introduced by amplification. Plasmids were banded on cesium chloride gradients twice prior to transfection.

Transfection of COS cells. COS cells were routinely transfected with L-containing plasmids at a confluence of 50 to 80% in 60-mm-diameter culture dishes. Supercoiled DNA (10 μg) was transfected by using Lipofectin (GIBCO-BRL) via the proprietary protocol. Cells were incubated for 24 h in Opti-MEM (GIBCO-BRL) to allow uptake of transfected DNA then incubated in Dulbecco's modified Eagle media supplemented with 10% fetal bovine serum for 48 h to allow expression of the L protein.

Preparation of cytoplasmic extracts. Cytoplasmic extracts which retained L activity were prepared as described for the isolation of cellular casein kinase II (33). Cells were washed three times with phosphate-buffered saline and then incubated on ice for 5 min in 1 ml of ice-cold hypotonic homogenization buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol). Cells were scraped into the buffer, transferred to a 7-ml Dounce homogenizer, and disrupted by 50 strokes. Cellular debris and nuclei were removed from the extract by centrifugation at 13,000 rpm at 4°C for 30 s. Extracts, which contained 0.2 to 0.6 μg of total cell protein per μl, were aliquoted and stored at −80°C.

VSV RNA synthesis in vitro. The N-RNA template for in vitro transcription was isolated from purified virions, and recombinant P(I) was purified from *E. coli* harboring an expression plasmid containing P(I) cDNA (3). Transcription reaction mixtures (20 μl) contained 0.5 to 5 μg of cytoplasmic extract, 40 U of RNase inhibitor, 50 ng of actinomycin D, 2 μg of N-RNA, and 25 ng of bacterial P protein in VSV transcription buffer (50 mM Tris-HCl [pH 8], 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM each ATP, GTP, and UTP, 50 μM CTP). Mixtures were incubated for 180 min at 30°C and then processed as described previously (3). RNA synthesis was monitored by the incorporation of [^{32}P]CTP and was visualized by autoradiography of 6% denaturing polyacrylamide gels. RNAs were quantified by using a Bio-Rad model 620 densitometer and IID Analyst software. Extracts retained transcriptional activity for 2 to 4 weeks, although assay of L mutants for transcription was routinely done within 1 week of preparation.

Western immunoblotting. L protein synthesis in transfected cells was monitored by Western blot analysis using a primary rabbit antibody to a synthetic peptide corresponding to the amino-terminal 5 to 19 residues of the L protein of VSV(I), which was also generously provided by Manfred Schubert. Total protein (40 μg) of each cytoplasmic extract was fractionated on a 7.5% denaturing polyacrylamide gel and then transferred to GeneScreen-Plus (NEN-DuPont). After blocking and probing with the primary antibody, membranes were probed with peroxidase-linked goat anti-rabbit immunoglobulin G and the complex was detected by enhanced chemiluminescence (Amersham). L synthesis was quantified by densitometry of the X-ray film, and extracts were diluted with a cytoplasmic extract of mock-transfected COS cells such that both the concentrations of L protein and the total protein concentrations were equivalent.

RESULTS

Recombinant L protein transcribes VSV mRNAs in vitro. COS cells were transfected with the expression plasmid pSV-VSL1, which was previously shown by complementation studies to direct the synthesis of biologically active L protein (31). Crude cytoplasmic extracts were prepared from these cells and added to VSV transcription reactions in vitro that also contained the N-RNA template purified from virions and bacterial, recombinant P protein. Results are presented in Fig. 1.

No VSV mRNA synthesis was observed when viral N-RNA was incubated alone or with bacterial P(I), indicating that the template did not contain detectable amounts of L protein. No RNA synthesis was observed when N-RNA was incubated with P protein together with extracts of either mock-transfected cells or cells transfected with the expression vector pJC119, which did not contain L cDNA. However, addition of extracts from cells transfected with pSV-VSL1 to the N-RNA and P protein resulted in the efficient synthesis of VSV mRNAs. Alignment of these transcripts with RNAs synthesized by disrupted virus particles (not shown) indicated that they correspond in size to, and presumably are, the mRNAs for the G, N, P, and M proteins of VSV. VSV mRNA synthesis did not occur when N-RNA was incubated with extracts of cells transfected with L protein but without bacterial P protein (data not shown). These results confirm that the L protein of VSV is synthe-

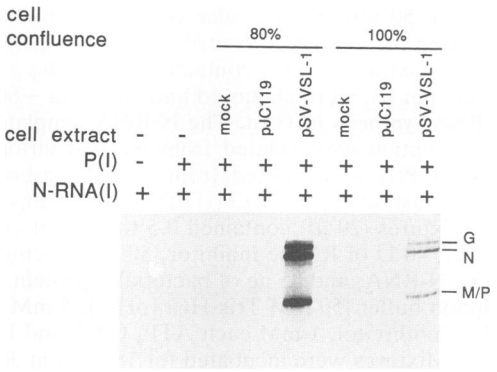

FIG. 1. Reconstituted VSV transcription using recombinant L and P proteins. Cells were transfected when 80 or 100% confluent. Reaction mixtures contained 300 ng of N-RNA purified from virus, with or without 25 ng of P(I) purified from bacteria or 0.5 μg of protein equivalent of cytoplasmic cell extract. After incubation for 3 h at 37°C, the poly(A) tails of nascent transcripts were removed by treatment with RNase H, and then the transcripts were purified by phenol-chloroform extraction and precipitation with isopropanol. [^{32}P]CTP-labeled transcripts were analyzed on a 6% denaturing polyacrylamide gel, which was exposed for 7 days.

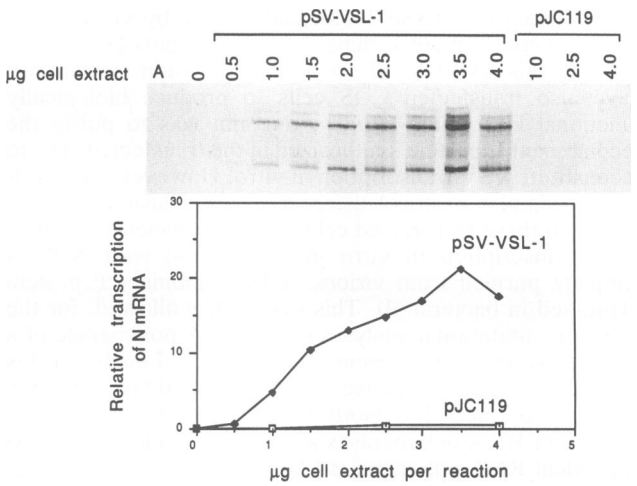

FIG. 2. Dose-response of VSV transcription with increasing amounts of cytoplasmic extract from cells expressing L protein. Also shown is the effect of adding extracts of cells transfected with the vector (pJC119) alone to the transcription reactions. Transcripts were analyzed as described in the text, and the gel was exposed for 48 h. RNA synthesis was quantified by densitometry.

sized in sufficient quantities in transfected COS cells to not only complement in vivo replication of L-deficient VSV mutants as observed previously (31) but also support in vitro RNA synthesis.

In these preliminary experiments, COS cells were transfected at various confluences. Expression of L protein in terms of in vitro RNA synthesis was most efficient when cells were 60 to 80% confluent and was greatly decreased when cells were 100% confluent (Fig. 1), presumably because transfection efficiency with Lipofectin decreases with increasing cell confluence (proprietary notes).

Synthesis of VSV mRNAs is proportional to the amount of cell extract used. The transcription reactions described so far contained 1 μg of each cytoplasmic extract. In another set of experiments, incubations containing increasing amounts of cell extract were used to determine the optimum concentration of cytoplasmic extract (Fig. 2). VSV RNA synthesis was proportional to the concentration of cell extract added up to 4 μg of protein per 20-μl reaction, which was used in subsequent reactions. Surprisingly, transcription at this relatively high concentration of cell extract was not inhibited by the addition of cellular RNases or proteases. Addition of increasing amounts of extract from cells transfected with pJC119 did not result in VSV mRNA synthesis.

Another experiment (data not shown) in which the amount of L protein was increased but the amount of total cellular protein remained the same resulted in a similar titration curve.

Sequence comparison of paramyxovirus and rhabdovirus L proteins. The protocol described for the reconstitution of VSV transcription in vitro using recombinant L protein allows, for the first time, systematic delineation of the various catalytic functions of the L protein by site-directed mutagenesis. Our first objective was to examine the role of a GDNQ motif that is conserved in all nonsegmented negative-strand viruses in terms of both sequence and position within the molecule. The primary structures of the L proteins of a number of rhabdoviruses and paramyxoviruses have been determined to date, including: those of the Indiana (30) and New Jersey (5, 12) serotypes of VSV, rabies virus (35), Sendai virus (32), Newcastle disease virus (36), measles virus (6), human parainfluenza viruses (24) and 3 (14), simian virus 5 (25), and sonchus yellow net virus (7). The role of the GDNQ motif, which is conserved in all of these viruses, has been the focus of considerable speculation.

Comparison of the amino acid sequences of many RNA-dependent RNA and DNA polymerases has revealed some regions of homology (23, 29). In particular, plus-strand RNA viruses contain a conserved GDD motif located within a region of hydrophobic residues which is probably involved in template recognition or binding or in phosphodiester bond formation. The L proteins of most negative-strand nonsegmented RNA viruses do not contain a GDD triplet; if there is one, there is no conservation of this motif with respect to position within the molecule or positions of surrounding hydrophobic residues. However, a GDNQ sequence is conserved in all paramyxoviruses and rhabdoviruses examined to date (28) (Fig. 3), and most reports of the sequences of L genes (reviewed in references 25 and 28) have suggested that this is a variant of the GDD motif of plus-strand RNA viruses (23). We have examined this proposal experimentally by using a series of mutations of the VSV L protein.

Site-directed mutagenesis of the VSV L protein. The conserved GDNQ motif of the L protein of VSV(I) is located between amino acid residues 712 and 715. We amplified a 303-nucleotide fragment of the L gene containing various mutations and introduced these mutations into the L gene by using the unique *Dra*III and *Sac*I sites of pSV-VSL1. The *Dra*III site includes the codon encoding the G of the conserved motif, so this position remained invariant throughout this study. However, we constructed a series of mutations which modified the conserved region from the aspartic acid residue onward. These mutations are shown in Fig. 4.

Comparison of the amino acid sequences of rhabdoviruses and paramyxoviruses within this region (Fig. 3) reveals that there is also considerable sequence conservation outside of the conserved GDN motif. We therefore took two approaches to the systemic mutational analysis of this region: (i) we introduced conserved and nonconserved amino acid changes at sites of interest, and (ii) we introduced mutations

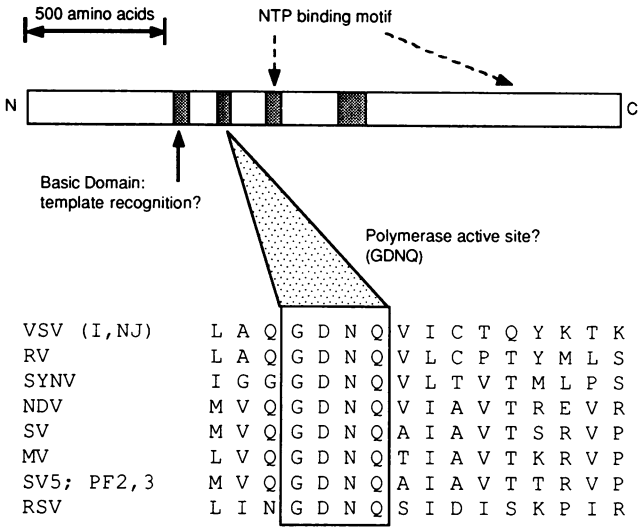

FIG. 3. Sequence comparison of the conserved GDNQ motifs and flanking sequences of rhabdoviruses and paramyxoviruses. The region shown corresponds to amino acids 710 to 725 of the L protein of VSV(I). Also shown are the relative positions of the GDNQ and other conserved blocks (5, 23, 28) within L(I). RV, rabies virus; SYNV, sonchus yellow net virus; NDV, Newcastle disease virus; SV, Sendai virus; MV, measles virus; SV5, simian virus 5; PF2,3, human parainfluenza virus 2 and 3; RSV, respiratory syncytial virus.

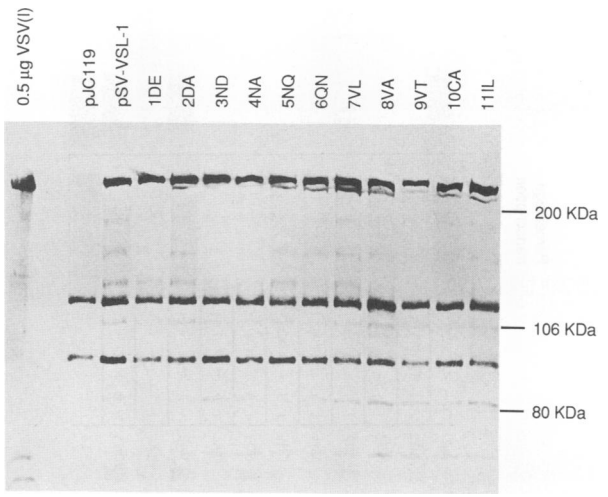

FIG. 5. Detection of L protein synthesis in transfected cells by Western blotting and enhanced chemiluminescence. The equivalent of 20 μg of total protein of each transfected cell extract was fractionated on a 7.5% polyacrylamide gel, electroblotted onto GeneScreen (Amersham), and probed with a rabbit antibody against the amino termini of the L protein. L protein was detected by enhanced chemiluminescence by using a peroxidase-linked goat anti-rabbit secondary antibody. The positions of protein size markers are indicated at the right.

that altered the VSV sequence at specific residues such that it resembled the sequences of other rhabdoviruses or paramyxoviruses. The latter mutations were made in the hope of discerning potential evolutionary relationships between the different viruses by virtue of retention of transcriptional activity.

Eleven mutations of the VSV L protein within the conserved GDNQ motif and downstream of this sequence were constructed (Fig. 4). Each mutant was transfected and examined for transcription in at least three separate experiments. Figure 5 shows a Western blot of each cytoplasmic extract probed with an antibody to a synthetic peptide corresponding to the carboxy terminus of L(I). Synthesis of L protein was quantified by densitometry of the Western blot (Fig. 5). Each extract was mixed with the extract of mock-transfected cells such that the final transcription reactions contained both the same amount of total soluble protein and the same amount of mutant or parental L protein. RNA synthesis was assayed, and the results were collated graphically by densitometry of the autoradiograph (Fig. 6).

Parental	L	A	Q	G	D	N	Q	V	I	C
1DE	-	-	-	-	E	-	-	-	-	-
2DA	-	-	-	-	A	-	-	-	-	-
3ND	-	-	-	-	-	D	-	-	-	-
4NA	-	-	-	-	-	A	-	-	-	-
5NQ	-	-	-	-	-	Q	-	-	-	-
6QN	-	-	-	-	-	-	N	-	-	-
7VL	-	-	-	-	-	-	-	L	-	-
8VA	-	-	-	-	-	-	-	A	-	-
9VT	-	-	-	-	-	-	-	T	-	-
10CA	-	-	-	-	-	-	-	-	-	A
11IL	-	-	-	-	-	-	-	-	L	-

FIG. 4. Single amino acid mutants of the L protein of VSV(I) constructed within and flanking the conserved GDNQ motif.

All VSV mutants directed the synthesis of full-length L proteins in transfected cells, determined by immunoreaction against antibodies directed against a synthetic peptide corresponding to the amino termini (Fig. 5) of VSV. Failure of a particular mutant to transcribe cannot, therefore, be attributed to defective synthesis of L protein due to deletion or introduction of a stop codon outside of the PCR-amplified region. Mutants which did not support VSV transcription in vitro were tested by using plasmid preparations grown from two separately isolated clones.

All mutations of the D or N residue of the core GDN abrogated RNA synthesis except one. Mutant L3ND contained a D residue in place of the N within the GDN motif and retained 27% transcriptional activity compared with the parental L protein. The observation that a recombinant L protein containing GDD is transcriptionally active is strong evidence that the GDN motif of these viruses represents a variant of the GDD of positive-strand RNA viruses. Moreover, the highly conserved Q residue of the GDNQ sequence must remain invariant, since a conserved mutation (Q to N) retained only about 5% of the transcriptional activity of the parental L protein.

A number of changes were made in the three amino acids downstream of the GDNQ, which show little conservation between different rhabdoviruses and paramyxoviruses. All such mutations retained transcriptional activity whether they were conserved changes, nonconserved changes, or changes which reflected the amino acid sequences of other viruses. However, the extents of transcriptional activity of these mutants varied. For example, mutation of the valine downstream of the GDNQ to alanine, as seen in Sendai virus, simian virus 5, and human parainfluenza viruses 2 and 3, retained full transcriptional activity. In contrast, mutation of this valine to a leucine or a threonine (as in measles virus) resulted in considerably less transcriptional activity.

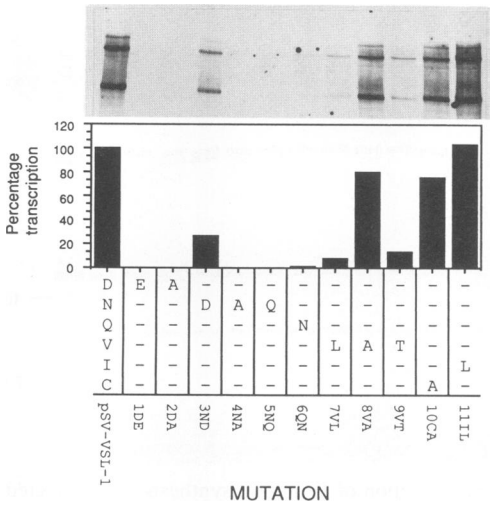

FIG. 6. Synthesis of VSV mRNAs in vitro by parental and recombinant VSV L proteins. Equal amounts of each cell extract containing equal amounts of L protein were used in VSV transcription reconstitution reactions as described in the text. mRNAs were quantified by densitometry of the autoradiograph, which was the result of a 24-h exposure. The position of each mutation between amino acids 714 (D) and 719 (C) is indicated below the histogram.

DISCUSSION

We have described a protocol for the synthesis of transcriptionally active, recombinant L protein of VSV. This is not the first report of a recombinant L protein of a negative-strand RNA virus; the L protein of VSV(I) has been expressed in vivo by rescuing VSV mutants (31) or replicating defective interfering particles (26, 27), and the L protein of the segmented, negative-strand Bunyamwera virus has been shown to replicate one of the genomic RNAs when expressed in vivo (22). One advantage of the system described here over these other protocols, which rely on viral or subviral replication as an assay for L protein function, is that the individual catalytic functions of the L protein can be delineated more easily in vitro. For example, a mutation that abrogates capping but not RNA synthesis per se could be introduced into a recombinant L protein. Such a lesion could be characterized in vitro but would probably simply result in failure of the virus (or subviral particle) to replicate in an in vivo assay. Moreover, a technical advantage of the protocol described here over other systems for examining L proteins is that the SV40 expression vector was found to be quite sufficient for the synthesis of enough L protein to support in vitro transcription; the rather more complex vaccinia virus expression system (13), which introduces another virus and its associated polymerase into the picture, is not required.

Structure-function analyses of the large, multifunctional L protein have been initiated by mutational studies of a GDNQ sequence which is conserved in negative-strand, nonsegmented RNA viruses. Results have largely confirmed that the GDN motif is a variant of the GDD of positive-strand RNA viruses (23), a prediction which has been made previously on the basis of several sequence comparisons. Little sequence conservation was required outside of the GDNQ motif for transcription. Perhaps the strict sequence homology of the three VSV L proteins sequenced to date (NJ_{Ogden}, $NJ_{Hazelhurst}$, and $I_{Mudd\text{-}Summers}$) flanking the GDNQ reflects a structural requirement for a cotranscriptional catalytic event, e.g., polyadenylation or capping.

Mutational analysis of the YGDD motif of the RNA polymerase gene of poliovirus (21) and Bunyamwera virus (22) obtained results similar to those described here; i.e., although very few mutations within the GDD motif were compatible with transcriptional activity, not all of the mutations were lethal. In contrast, any modification of the YGDD of Qβ resulted in loss of transcription (20). Our observation that the VSV L protein is transcriptionally active when it contains GDD in place of GDN suggests that, conversely, the polymerase of poliovirus (and other plus-strand RNA viruses) might function with a GDN motif. In the case of Bunyamvera virus, however, the recombinant L protein is not capable of supporting viral replication when the native SDD polymerase domain is converted to GDN (22). The results presented here provide further support for the previous studies of poliovirus, Bunyamwera virus, human immunodeficiency virus, and Qβ bacteriophage, which concluded that the GDD motif plays a central role in RNA synthesis, perhaps by binding metal ions (9, 23, 29), and may represent the enzyme active site. It is not obvious why the nonsegmented, negative-strand RNA viruses have evolved a GDN sequence in place of GDD; this difference may simply reflect the increased transcription observed with GDN compared with GDD.

It is worth noting that in structural models of the GDD-containing core region of the Klenow fragment of *E. coli* polymerase I (9), the first aspartate residue is integral to the catalytic active site of the molecule. In the present study, any mutation of this aspartate residue resulted in a lethal mutation (GDN to GEN or GAN), providing support for this proposal. In contrast, mutations of the second aspartate, as observed here, or the glycine (21) are not necessarily incompatible with RNA synthetic activity. We have not yet determined whether mutation of the glycine of the GDNQ motif can be tolerated with respect to transcription, as this residue forms part of the unique restriction enzyme site used to construct the mutations. Future studies will focus upon this glycine and conserved amino acids upstream of the GDNQ motif.

One possibility that cannot be directly excluded is that the mutations made within the L protein adversely affect the interaction of the recombinant L protein with either the N-RNA template or the P protein. However, this does seem unlikely given that the only mutation of the core GDN that retained transcriptional activity was the mutation to GDD.

In this study, we have provided experimental evidence that the conserved GDN of rhabdoviruses and paramyxoviruses is the equivalent of the GDD motif of plus-strand RNA viruses, using a novel protocol for the synthesis of recombinant VSV L protein that will support RNA synthesis in vitro. This protocol will allow the synthesis of transcriptionally active L protein for the study of the P and N proteins in transcription; previously, such studies involved the purification of L protein from virions, which is both laborious and inconsistent in terms of the purity and activity of the L protein. Expression of transcriptionally active L protein in transfected cells will also further investigation of the other properties which have been tentatively assigned to the L protein of VSV. One of the most disputed properties assigned to the L protein is its ability to phosphorylate the P protein and the role played by any such phosphorylation in RNA synthesis. Sequence comparisons of rhabdovirus and paramyxovirus L proteins have identified a conserved region that resembles a consensus ATP-binding motif that may be one of the two distinct nucleoside triphosphate (NTP)-binding sites demonstrated in VSV(NJ) (16, 17). Our studies can now address the role of this potential NTP-binding motif

(and others within the L protein) in transcription and phosphorylation of the P protein.

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REFERENCES

1. **Baltimore, D., A. S. Huang, and M. Stampfer.** 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci. USA **66**:572–576.
2. **Banerjee, A. K., and S. Barik.** 1992. Gene expression of vesicular stomatitis virus genome RNA. Virology **188**:417–428.

2a. **Barik, S.** Unpublished data.

3. **Barik, S., and A. K. Banerjee.** 1991. Cloning and expression of the vesicular stomatitis virus phosphoprotein gene in *Escherichia coli*: analysis of phosphorylation status versus transcriptional activity. J. Virol. **65**:1719–1726.
4. **Barik, S., and A. K. Banerjee.** 1992. Sequential phosphorylation of the phosphoprotein of vesicular stomatitis virus by cellular and viral protein kinases is essential for transcription activation. J. Virol. **66**:1109–1118.
5. **Barik, S., E. W. Rud, D. Luk, A. K. Banerjee, and C. Yong Kang.** 1990. Nucleotide sequence analysis of the L gene of vesicular stomatitis virus (New Jersey serotype): identification of conserved domains in L proteins of nonsegmented negative-strand RNA viruses. Virology **175**:332–337.
6. **Blumberg, B. M., J. C. Crowley, J. I. Silverman, J. Menonna, S. D. Cook, and P. C. Dowling.** 1988. Measles virus L protein evidences elements of ancestral RNA polymerase. Virology **164**:487–497.
7. **Choi, O., S. Kuwata, E. V. Koonin, L. A. Heaton, and A. O. Jackson.** 1992. Structure of the L (polymerase) protein gene of sonchus yellow net virus. Virology **189**:31–39.

7a. **Das, T., and A. K. Banerjee.** Expression of the vesicular stomatitis virus nucleocapsid protein gene in Escherichia coli: analysis of its biological activity in vitro. Virology, in press.

8. **De, B. P., and A. K. Banerjee.** 1984. Specific interactions of L and NS proteins of vesicular stomatitis virus with heterologous genome ribonucleoprotein template lead to mRNA synthesis in vitro. J. Virol. **51**:628–634.
9. **Delarue, M., O. Poch, N. Tordo, D. Moras, and P. Argos.** 1990. An attempt to unify the structure of polymerases. Protein Eng. **3**:461–467.
10. **Emerson, S. U., and R. R. Wagner.** 1973. L protein requirements for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **12**:1325–1335.
11. **Emerson, S. U., and Y. H. Yu.** 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **15**:1348–1356.
12. **Feldhaus, A. L., and J. A. Lesnaw.** 1988. Nucleotide sequence of the L gene of vesicular stomatitis (New Jersey): identification of conserved domains in the New Jersey and Indiana L proteins. Virology **163**:359–368.
13. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
14. **Galinski, M. S., M. A. Mink, and M. W. Pons.** 1988. Molecular cloning and sequence analysis of the human parainfluenza virus gene encoding the L protein. Virology **165**:499–510.
15. **Gill, D. S., D. Chattopadhyay, and A. K. Banerjee.** 1986. Identification of a domain within the phosphoprotein of vesicular stomatitis virus that is essential for transcription in vitro. Proc. Natl. Acad. Sci. USA **83**:8873–8877.
16. **Hammond, D. C., R. K. Evans, and J. A. Lesnaw.** 1992. The L protein of vesicular stomatitis virus transcription complexes is specifically photolabelled by 5-azido-uridine 5′-triphosphate, an analogue of the RNA polymerase substrate uridine 5′-triphosphate. J. Gen. Virol. **73**:61–66.
17. **Hammond, D. C., B. E. Haley, and J. A. Lesnaw.** 1992. Identification and characterization of the serine/threonine protein kinase activity intrinsic to the L protein of vesicular stomatitis virus. J. Gen. Virol. **73**:67–75.
18. **Horikami, S. M., and S. A. Moyer.** 1982. Host range mutants of vesicular stomatitis virus defective in *in vitro* RNA methylation. Proc. Natl. Acad. Sci. USA **79**:7694–7698.
19. **Hunt, D. M., E. G. Smith, and D. W. Buckley.** 1984. Aberrant polyadenylation by a vesicular stomatitis mutant is due to an altered L protein. J. Virol. **52**:515–521.
20. **Inokuchi, V., and A. Hirashima.** 1987. Interference with viral infection by defective RNA replicase. J. Virol. **61**:3946–3949.
21. **Jablonski, S. A., M. Luo, and C. D. Morrow.** 1991. Enzymatic activity of poliovirus RNA polymerase mutants with single amino acid changes in the conserved YGDD amino acid motif. J. Virol. **65**:4565–4572.
22. **Jin, H., and R. M. Elliott.** 1992. Mutagenesis of the L protein encoded by Bunyamwera virus and production of monospecific antibodies. J. Gen. Virol. **73**:2235–2244.
23. **Kamer, G., and P. Argos.** 1984. Primary structural comparison of RNA-dependent RNA polymerases from plant, animal and bacterial viruses. Nucleic Acids Res. **12**:7269–7282.
24. **Kawano, M., K. Okamoto, H. Bando, K. Kondo, M. Tsurudome, H. Komada, M. Nishio, and Y. Ito.** 1991. Characterization of the human parainfluenza type 2 virus gene encoding the L protein and the intergenic sequences. Nucleic Acids Res. **19**:2739–2746.
25. **Parks, G. D., C. D. Ward, and R. A. Lamb.** 1992. Molecular cloning of the NP and L genes of simian virus 5: identification of highly conserved domains in paramyxovirus NP and L proteins. Virus Res. **22**:259–279.
26. **Pattnaik, A. K., and G. W. Wertz.** 1990. Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. J. Virol. **64**:2948–2957.
27. **Pattnaik, A. K., and G. W. Wertz.** 1991. Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly and budding of defective interfering particles. Proc. Natl. Acad. Sci. USA **88**:1379–1383.
28. **Poch, O., B. M. Blumberg, L. Bougueleret, and N. Tordo.** 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J. Gen. Virol. **71**:1153–1162.
29. **Poch, O., I. Sauvaget, M. Delarue, and N. Tordo.** 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J. **8**:3867–3874.
30. **Schubert, M., G. G. Harmison, and E. Meier.** 1987. Primary structure of the vesicular stomatitis virus polymerase (L) gene: evidence for a high frequency of mutations. J. Virol. **51**:505–514.
31. **Schubert, M., G. G. Harmison, C. D. Richardson, and E. Meier.** 1985. Expression of a cDNA encoding a functional 241-kilodalton vesicular stomatitis RNA polymerase. Proc. Natl. Acad. Sci. USA **82**:7984–7988.
32. **Shioda, T., K. Iwasaki, and H. Shibuta.** 1986. Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins. Nucleic Acids Res. **14**:1545–1563.
33. **Sleat, D. E., N. Chikkala, S. Gautam, and A. K. Banerjee.** 1992. Restricted replication of vesicular stomatitis virus in T-lymphocytes is coincident with a deficiency in a cellular protein kinase required for viral transcription. J. Gen. Virol. **73**:3125–3132.
34. **Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini.** 1983. The expression of a recombinant DNA gene coding for the vesicular stomatitis virus nucleocapsid protein. J. Virol. **45**:773–781.
35. **Tordo, N., O. Poch, A. Ermine, G. Keith, and F. Rougeon.** 1988. Completion of the rabies virus genome sequence determination: highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. Virology **165**:565–576.
36. **Yussoff, K., N. S. Millar, P. Chambers, and P. T. Emmerson.** 1987. Nucleotide sequence analysis of the L gene of Newcastle disease virus: homologies with Sendai and vesicular stomatitis virus. Nucleic Acids Res. **15**:3961–3976.