Normal Replication of Vesicular Stomatitis Virus without C Proteins

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The expression of two small basic proteins (C and C∗) encoded by a second open reading frame of the New Jersey serotype of vesicular stomatitis virus (VSV) P gene was reported previously (Spiropoulou and Nichol, J. Virol., 67, 3103 – 3110, 1993). Here we found that the Indiana serotype virus also expressed C and C∗ proteins from this reading frame. We eliminated C and C∗ expression by making a single base change that introduced a stop codon in the C and C∗ coding sequence, but left the P-protein sequence unchanged. This mutated P gene supported normal replication and packaging of VSV minigenomes encoding G and M proteins. The mutated P gene was also recombined into an infectious clone of VSV that was used to recover virus. The mutant virus no longer expressed the C and C∗ proteins but showed growth kinetics identical to wild-type virus. The amounts of viral mRNAs and proteins synthesized were indistinguishable in mutant and wild-type virus infected cells as were the yields and composition of mutant and wild-type virus particles. The kinetics of host protein-synthesis shut-off were also identical for both viruses. Although the C and C∗ proteins were dispensable for VSV growth in tissue culture, they are known to be conserved in all vesiculoviruses, and thus perhaps play a role in viral pathogenesis or transmission by insect vectors.

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INTRODUCTION

The use of overlapping reading frames to generate more than one unique protein from a single mRNA has been described for several viruses. The synthesis of influenza B virus NA and NB glycoproteins and Sendai virus P and C proteins from overlapping frames were among the first well-characterized examples (Giorgi et al., 1983; Shaw et al., 1983). Since then, numerous other examples have been found, the most striking of which is the existence of multiple functional open reading frames within the P mRNA of several paramyxoviruses (Curran and Kolakofsky, 1990; Lamb and Paterson, 1991). A similar phenomenon has been recently described for another family of negative-strand RNA viruses, the Rhabdoviridae (Spiropoulou and Nichol, 1993).

Rhabdoviruses are membrane-enveloped viruses that are widely distributed in nature where they infect vertebrates, invertebrates, and plants. The two genera of Rhabdoviridae are the vesiculoviruses (vesicular stomatitis virus (VSV)-like) and lyssaviruses (rabies-like). These viruses have single, negative-strand RNA genomes of approximately 11,000 nucleotides (Schubert and Rose, 1987). Because the genome is the negative sense, rhabdoviruses must encode and package an RNA-dependent RNA polymerase in the virion (Baltimore et al., 1970). This enzyme transcribes genomic RNA to make subgenomic mRNAs that encode viral proteins and it also replicates full-length positive-sense and negative-sense RNAs. As polymerase proceeds along the genome during transcription, it probably terminates after polyadenylating each mRNA. After termination, the polymerase initiates with only 70–80% frequency, resulting in a transcription gradient (attenuation) that follows the gene order (Iverson and Rose, 1981). Although this model is generally accepted, there are no data that rule out a cleavage model for generation of VSV transscripts.

The prototype rhabdovirus, VSV, has five genes that are transcribed in the order N-P-M-G-L. The proteins encoded by these genes are all present in the virion. However, the VSV P gene from the New Jersey serotype has recently been shown to encode two additional highly basic proteins of 55 and 65 amino acids which are generated from the same mRNA by alternative translational initiation in a second reading frame (Spiropoulou and Nichol, 1993). These proteins called C and C∗, were detected using anti-peptide antibodies raised against potential open reading frame (ORF) products and can be immunoprecipitated from lysates of VSV New Jersey infected cells. Potential to encode these small highly basic proteins is conserved among the vesiculoviruses but not in lyssaviruses. Conservation of this reading frame suggests an important but unknown function in viral replication. The P-gene sequence of the VSV Indiana serotype virus also contains a second ORF between nucleotides 84 and 284. Depending on which start codon is used,
this sequence could express two proteins of 67 and 55 amino acids (Spironoulou and Nichol, 1993).

In this study we were interested in determining if the VSV serotype Indiana also encodes these C and C' proteins from the P mRNA. Furthermore, we wanted to analyze the possible function of these proteins in the replication of VSV. Recently available systems allow derivation of replicating and transcribing VSV minigenomes from DNA copies (Stillman et al., 1995) or rescue of complete VSV from DNA (Lawson et al., 1995; Whelan et al., 1995). These systems were used to study the importance of C proteins in VSV replication. Surprisingly, we find that expression of C proteins is not important for replication of minigenomes or of complete VSV.

**MATERIALS AND METHODS**

**Plasmid constructions**

A VSV P gene containing a stop codon in ORF2 was constructed as follows. In the initial step, we used primers 5'-AGCTCGAGTGAAGAGCATAC and 5'-AATTC-GTCAAGCTTCTGGATCTGG to amplify a 112-bp fragment from the VSV P gene. The first primer introduced a point mutation (G145 to A [in bold]) which generates a stop codon for the second open reading frame but only a silent mutation for the first open reading frame encoding P (Fig. 1). The PCR product was digested with Earl and Cell and cloned back into the VSV-P gene. For this purpose, the VSV-P gene in pBluescript(SK+) was digested with EcoRV and Cell and ligated to an EcoRV-Earl fragment containing the first 140 nucleotides at the 5' end of VSV-P and the PCR fragment. The sequences within the resulting plasmid generated by PCR were determined and found to contain the desired mutation. The expression of the P gene was then checked by using the vaccinia T7 system (Fuerst et al., 1986). The plasmid was designated pBSP-C'.

To introduce the mutation into the clone of the complete VSV genome, an EcoRV-EspI site from the mutant VSV-P gene was isolated and subcloned into the EcoRV-EspI sites of a plasmid containing both VSV-P and -N genes. A Bst1107I-Xbal fragment containing partial 3' sequences of the VSV-N and partial 5' sequences of the VSV-P genes was then cloned into the Bst1107I-Xbal sites of a plasmid encoding the complete VSV genome (Lawson et al., 1995) to generate pVSVC-. The presence of the mutation was verified by sequence analysis performed by the Yale University oligonucleotide synthesis service.

A plasmid for expression of C' protein from a bacterial promoter was constructed by cloning the coding sequence into the vector pQE30 (QIAGEN) which produces a C'-fusion protein with 6 histidine residues at its aminoterminus. To accomplish this, we used primers 5'-CGCGGGATCCCATGAGATCGAAGAC and 5'-CGCGC-GAGCTAGGGTTTTC to amplify a PCR fragment from P mRNA by RT-PCR. The PCR product was digested with BamHI and Sacl (underlined) and cloned into the BamHI/Sacl sites of plasmid pQE30. The plasmid was designated pQE30-C'.

**Antiserum**

*Escherichia coli* were transfected with pQE30-C' and C' expression was induced for 2 hr with IPTG according to the directions supplied (QIAGEN). Following purification using nickel agarose affinity chromatography, the protein was used to immunize a rabbit in complete Freund's adjuvant. IgG was purified from the serum by protein G-agarose chromatography.

**Minigenome passaging**

Baby hamster kidney cells (BHK-21, ATCC) were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% FBS. Cells on coverslips in 35-mm-diameter dishes were infected with vTF7-3 (Fuerst et al., 1986) at a multiplicity of 10 PFU/cell. After 30 min, the plasmid GM(MG containing both the G and M protein genes (MG stands for minigenome) and N, P, and L proteins or N, P-C', and L proteins were transfected into the cells using TransfectACE (Rose et al., 1991). The coding regions of the plasmids were each expressed in pBluescript SK(+) from the T7 promoter. The ratios and protocols used were the same as those described earlier (Stillman et al., 1995). After 24 hr incubation at 37°C, the culture supernatants were collected and the cells fixed in 3% paraformaldehyde and processed for immunofluorescence as described earlier (Lawson et al., 1995). The M and G genes in the construct are functional, allowing passaging of minivirus in cells expressing N, P, and L proteins. To passage the miniviruses, the supernatants from these cells were plated onto BHK cells transfected with plasmids encoding N, P, and L or N, P-C', and L proteins only.

**Transfection and recovery of mutated recombinant VSV**

Transfection and recovery of mutant virus was performed by modifications of previously described methods (Lawson et al., 1995). BHK cells plated on 10-cm dishes were infected with vTF7-3 at a multiplicity of infection of 10. After 60 min, the plasmids encoding the mutant VSV antigenomic RNA and the N, mutant P, and L proteins were transfected into the cells by using calcium phosphate transfection according to directions supplied (GIBCO). The coding regions for N, P-C', and L proteins were each expressed in pBluescript (SK+) from the T7 promoter. Plasmid amounts were 10 μg of pVSVC-, 3 μg of pBS-N, 5 μg of pBSP-C', and 2 μg of pBS-L. After 48 hr incubation, supernatants were removed and centrifuged for 10 min at 1250 g. Four milliliters of the supernatant was then added to ~5 × 10⁶ BHK cells on a 6-cm
Preparation and analysis of VSV protein and RNA

Recombinant wild-type VSV and mutant VSV isolated from a single plaque were used to infect BHK cells (≈60% confluent) at 0.1 PFU/cell on a 10-cm dish in 10 ml of DMEM/5% fetal bovine serum. After 24 hr, cell debris and nuclei were removed by centrifugation at 1250 g for 5 min and virus was pelleted from the supernatant by centrifugation through a 10% sucrose cushion at 35,000 rpm for 1 hr, using a Beckman SW41 rotor. Virus pellets were resuspended in 10 mM Tris- HCl, pH 7.4, for protein analysis.

For metabolic labeling of the VSV proteins, BHK cells on a 35-mm dish (≈70% confluent) were infected with wild-type or mutant VSV at a multiplicity of 10 PFU/cell. After 4 hr cells were washed with methionine-free DMEM and incubated for 1 hr at 37°C in 0.5 ml methionine-free DMEM containing 60 μCi of [35S]methionine. Cell extracts were analyzed by SDS–PAGE (10 or 25% acrylamide) and detected on a Phosphorimager (Molecular Dynamics) or by fluorography.

For immunoprecipitation, infected BHK cells were lysed in RIPA buffer. C proteins were immunoprecipitated using the polyclonal C’ antibody as described before (Spiropoulou and Nichol, 1993).

For RNA isolation, virions were treated with detergent and their RNA precipitated as described earlier (Lawson et al., 1995). For analysis of RNA by RT–PCR, primer pairs flanking the mutated region were used. The first-strand DNA synthesis was done in 50 μl of PCR buffer containing 5 mM MgCl₂, 1 mM dNTPs, 1 unit of RNasin (PROMEGA), 0.75 μM primer, and −0.25 μg mutant and wild-type VSV genomic RNA. Incubation was at 42° for 15 min followed by 5 min at 99° and 5 min at 72°. PCR was accomplished by addition of 0.5 unit of Taq polymerase, adjustment of MgCl₂ concentration to 1.25 mM, and addition of the second primer (0.75 μM). The reaction was subjected to 20 thermal cycles: 92°, 1 min; 58°, 1.5 min; 72°, 2 min.

Northern blot analysis

Total RNA was isolated from 5 × 10⁶ BHK cells at 4 hr postinfection by TRIzol Reagent according to the instructions supplied (GIBCO). One microgram total RNA was analyzed by denaturing gel electrophoreses and Northern hybridizations using [³²P]-labeled pSVV (Lawson et al., 1995) generated by random priming (Boehringer Mannheim) as probes specific for N, P, M, G, and L.

**RESULTS**

Minigenome replication and transcription

To examine the function of the second reading frame in the VSVVP gene, we eliminated the coding potential by introducing a stop codon that would truncate the protein after 8 or 20 amino acids, depending on which initiation codon was used (Fig. 1). The mutant plasmid was designated pBSP-C−. The mutation did not affect the expression of an intact P protein, since it created only a silent mutation for the first open reading frame. To determine if these ORF2 proteins were required for VSV replication and assembly, we first used minigenomes encoding two VSV proteins (Stillman et al., 1995) because we suspected the mutation might be lethal to VSV replication. The DNA construct used (GM MG [MG stands for minigenome]) contained the G and M genes from VSV in tandem flanked by the trailer and leader regions from the wild-type VSV genome. The minigenome plasmid is transcribed by T7 RNA polymerase to generate a negative sense VSV RNA containing the G and M genes. When coexpressed with N, P, and L proteins, this RNA replicates and is transcribed to generate G and M mRNA and proteins. The expression of G and M is totally dependent on the expression of the support plasmids L, P, and N. Omission of any one completely abolishes replication. To determine if ORF2 expression was required for transcription and replication, P-C− instead of P was used as a support plasmid. GM MG, together with N, P, and L, or N, P-C−, and L as support plasmids were transfected into BHK cells. After 24 hr, expression of G was examined by immunofluorescence. The results showed that G was expressed to similar levels in experiments whether P or P-C− was used, suggesting that ORF2 products do not have an important function in replication of the minigenome (data not shown).

In the minigenome system, sufficient G and M proteins are produced to allow the assembly and release of mini-viruses from the transfection. Subsequent passaging onto cells expressing N, P, and L results in an amplification of the infectious particles. Therefore, this system is ideal for determining if proteins are important for the assembly of VSV. To determine if C proteins were essential for the assembly process, we passaged the infectious particles four times onto cells expressing N, P, and L or N, P-C−, and L. Immunofluorescence data (Fig. 2) showed that G was expressed similarly whether ORF2 proteins were present (Fig. 2A) or absent (Fig. 2B). These results suggest that ORF2 proteins are not crucial for the assembly and packaging of small infectious particles.

Recovery of VSV lacking P ORF2

These results above were obtained from a system using a truncated VSV genome. It was possible, however, that the ORF2 products might have an important
function in some aspect of VSV replication that was not mimicked by the minigenome system. Therefore, we used the system we developed for recovery of VSV from a complete DNA copy (Lawson et al., 1995). We constructed a full-length VSV clone in which the P gene was replaced with mutant P. The plasmid was designated pVSV-C \textsuperscript{0} and the presence of the point mutation was verified by sequencing.

The recovery of mutant VSV from cloned DNA involved simultaneous transfection of the plasmid encoding the full-length antigenomic RNA along with the three plasmids encoding VSV N, P-C \textsuperscript{0}, and L proteins in cells infected with the vaccinia virus vTF7-3 (Lawson et al., 1995; Fuerst et al., 1986). We decided to replace P with mutant P to avoid possible problems of recombination events which could occur between the full-length plasmid DNA and the support plasmids used in the initial recovery. Recovery of mutant VSV from the mutated DNA was successful, and we then proceeded to characterize the mutant virus. Subsequent plating on BHK cells revealed plaques from the mutant VSV virus that were detectable in <24 hr and indistinguishable from plaques recovered from the wild-type clone. An isolated plaque was used to grow stock virus. The virus grew to high titers (5 × 10\textsuperscript{9} PFU/ml), identical to wild-type VSV recovered from recombinant DNA (Lawson et al., 1995).

Identification of the point mutation

In pVSV-C \textsuperscript{0}, the VSV genome differs only in a single point mutation from wild-type VSV. To verify that this mutation had not reverted in recovered virus, we carried out reverse transcription of genomic RNA purified from mutant virions using primers upstream of the mutation. The reverse transcription product was then amplified by PCR using an additional primer downstream of the mutated site. No product was obtained when reverse transcriptase was omitted from the reaction before PCR, indicating that the PCR product was derived from RNA, not from contaminating DNA. Direct sequencing of the PCR product verified the presence of the mutation in this virus as well as in virus passaged one additional time.

Growth curve of mutant VSV

To determine if the replication cycle of the mutant virus differed from that of wild-type virus at earlier time points, a one-step growth curve was carried out for the mutant VSV and wild-type VSV. All the cells were infected simultaneously by using a high m.o.i. which facilitates a practically synchronous initiation of infection. Unadsorbed virus was washed away and the amount of virus released after different time points was determined by plaque assays on BHK cells (Fig. 3). The results show that wild-type and mutant virus replicate at the same rate. In this experiment, the mutant gave a slightly higher titer and in a second experiment the final wild-type titer was slightly higher, but no significant differences were noted between mutant and wild-type. Since we did not detect any difference in the virus growth, we decided to examine other aspects of virus replication in greater detail.

Analysis of viral proteins

To determine the level of protein expression from the mutant virus in infected BHK cells, we labeled wild-type

\[
\text{N} \begin{array}{c}
\text{P} \\
\text{C} \\
\text{C'}
\end{array}
\begin{array}{c}
\text{M}
\end{array}
\]

\[
\begin{array}{c}
\text{AAACAGATATCGAAATTCCTCAAAAGTTGAGTATCTCAAGGCTCTTCTGCATCGACGGGTAAGAGGA} \\
\text{MDNLTQKVPYELKYSYSRLDLGDAVSE}
\end{array}
\begin{array}{c}
P\rightarrow
\end{array}
\begin{array}{c}
\text{TATAGTCAGATCGACAGACGAGCTGAAATCTCCAAATTGAGTTGTTTCCCAAGGATGGAGGAGAAGAAAGCATCAGAC} \\
\text{IDEEIEAQRAEKSNYELFQEGDGYEEHTK}
\end{array}
\begin{array}{c}
C' \rightarrow
\end{array}
\begin{array}{c}
\text{MRSKHNELKSPIMSCSKRKMEWKSILS}
\end{array}
\begin{array}{c}
\text{C} \rightarrow
\end{array}
\begin{array}{c}
\text{STOP}
\end{array}
\begin{array}{c}
\text{CCCTCTTATTCTCAGGACGAGATGATCTGACACAAATTCTGAAACCAGAATTGGAAGGACATCAAGGCTGTATGCACCC} \\
\text{PSYFQADDSDESEPEIENQGAMYAP}
\end{array}
\begin{array}{c}
\text{PLIFNQMILTONLNLQKLKTAICMHI}
\end{array}
\begin{array}{c}
320
\end{array}
\begin{array}{c}
\text{AGATCCGAAACGTGAGCAGCTGGCTTTCTACACGGGCTTTCAGATGACTATGCAAGGAAATGAGATGTTGAT****} \\
\text{DPGEAQVEGFQIGQPLODYACEAEVQV****}
\end{array}
\begin{array}{c}
QIGKLKLKALKLYRSL**
\end{array}
\]

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the predicted C proteins. The first 320 nucleotides of the VSV-P gene Indiana serotype are presented. ORF2 begins at position 84 and terminates with the stop codon at position 284. The deduced amino acid sequence of ORF1 (P) and ORF2 (C and C') is indicated below the nucleotide sequence. The mutation changed G145 to A and introduced a stop codon into ORF2.
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FIG. 3. Kinetics of mutant virus growth. \( \approx 5 \times 10^4 \) BHK cells were infected (m.o.i. = 10) with wild-type (wt) or mutant (mu) VSV. Supernatant samples were collected at the indicated times postinfection and titers analyzed by plaque assays on BHK cells.

and relative amounts of the five viral proteins were indistinguishable in the wild-type and mutant virus.

C proteins are expressed in cells infected with wild-type, but not with mutant VSV

Because initially we did not have an antiserum recognizing the C proteins, we attempted to detect the C proteins directly by metabolic labeling of wild-type and mutant infected cells. When we analyzed lysates from [\(^{35}\)S]-

FIG. 2. Immunofluorescence of cells expressing G proteins from minigenomes. BHK cells were infected with vTF7.3 and then transfected with pBS-GM\(^{\text{MG}}\) and plasmids encoding N, P, and L or N, P, C\(^0\), and L proteins. The cells were fixed at 24 hr postinfection and probed for G protein on the cell surface. (A) Groups of cells expressing G protein on their surface. (B) Group of cells expressing G protein where P-C\(^-\) served as a support plasmid.

or mutant infected cells with 60 \( \mu \)Ci [\(^{35}\)S]methionine and analyzed crude lysates by SDS–PAGE. Because VSV infection shuts off host mRNA translation, the viral proteins can be visualized without immunoprecipitation. The results in Fig. 4 show that the L, G, N, P, and M proteins seen in wild-type virus infected cells (Fig. 4a) are all present in mutant infected cells also (Fig. 4b). The relative ratios of the proteins were the same, suggesting no effect of the mutation on transcription attenuation. Next, we analyzed the protein composition in the mature virions of the mutant virus. Virus pelleted from the supernatant was examined by SDS–PAGE. Figure 4 shows the Coomassie-stained gel of proteins present in wild-type (Fig. 4c) and mutant (Fig. 4d) VSV virions. Again, the mobilities

FIG. 4. Analysis of mutant and wild-type VSV protein in cells and virions. BHK cells were labeled 4 hr postinfection for 1 hr with 60 \( \mu \)Ci of [\(^{35}\)S]methionine in 0.5 ml of methionine-free medium and lysed in a detergent solution. Cell lysates from wild-type (a) or mutant VSV-infected (b) BHK cells were then analyzed by SDS–PAGE followed by fluorography. To analyze proteins from virions, 2% of the virions recovered from \( \approx 5 \times 10^6 \) BHK cells infected with either wild-type (c) or mutant VSV (d) were separated by SDS–PAGE and visualized by staining with Coomassie brilliant blue. Positions of the five VSV proteins are indicated.
Time course of host protein-synthesis shut-off

One possible role of the basic C proteins is shut-off of host protein synthesis. Although the data in Fig. 4 showed this was occurring, we were interested in determining if there were any differences in the kinetics of shut-off. BHK cells infected with wild-type or mutant VSV were pulse-labeled for 10 min with $[^{35}S]$methionine at several times postinfection and whole cell lysates were analyzed by SDS–PAGE (Fig. 7). The results show that the rate of mutant VSV protein production was the same as wild type and that there was no difference in the rate or extent of host protein-synthesis shut-off.

**DISCUSSION**

The ability of vesiculoviruses to encode C proteins from a second reading frame in the P gene has been known for several years (Spiropoulou and Nichol, 1993), but to date, there has been no examination of the C-protein function. Chattopadhyay and Banerjee (1988) had observed that the region of the VSV serotype New Jersey P gene encoding C proteins could be replaced with the amino terminus of $\beta$-tubulin and still support transcription in vitro. This suggested that C proteins do not play an essential role in transcription. Observations with VSV temperature-sensitive mutants possessing defects in the P gene showed that the ones defective in replication had nucleotide changes that would alter the C-protein amino acid sequence (Pringle, 1987; Rae and Elliot, 1986). Mutants with defects in transcription had nucleotide changes which would not alter the C-protein sequence. These findings led Spiropoulou and Nichol (1993) to speculate that the C proteins might function in RNA replication.

Information on function of potentially related C proteins from a distantly related negative strand RNA virus, Sen-
As predicted, the smaller protein was expressed at higher levels. These VSV Indiana C proteins were not present in virions, confirming the results reported for VSV New Jersey C proteins (Spiropoulou and Nichol, 1993).

Although the ORF2 proteins are dispensable for VSV replication in BHK cells, we cannot exclude the possibility that ORF2 products are important for replication in the natural host animal. Many other proteins thought to be nonessential actually play a role in countering the host immune system and thus play an important role in the pathogenesis of the virus (reviewed in Goording, 1992). It is conceivable that the ORF2 products might be immunomodulators or that they might play a role in replication in insect vectors and transmission to cattle. Future experiments will address these issues.

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