JOURNAL OF VIROLOGY, Mar. 1988, p. 826–833
0022-538X/88/030826-08$02.00/0

Vol. 62, No. 3

Rift Valley Fever Virus M Segment: Use of Recombinant Vaccinia Viruses To Study *Phlebovirus* Gene Expression

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Received 5 October 1987/Accepted 30 November 1987

Recombinant vaccinia viruses were constructed and used in conjunction with site-specific antisera to study the coding capacity and detailed expression strategy of the M segment of the *Phlebovirus* Rift Valley fever virus (RVFV). The M segment could be completely and faithfully expressed in recombinant RVFV-vaccinia virus-infected cells, the gene products apparently being correctly processed and modified in the absence of the RVFV L and S genomic segments. The proteins encoded by the RVFV M segment included, in addition to the viral glycoproteins G2 and G1, two previously uncharacterized polypeptides of 78 and 14 kilodaltons (kDa). By manipulation of RVFV sequences present in the recombinant vaccinia viruses and use of specific antibody reagents, it was found that the 78-kDa protein initiated at the first initiation codon of the open reading frame and encompassed the entire preglycoprotein and glycoprotein G2 coding sequences. The 14-kDa protein appeared to begin from the second in-phase ATG and was composed of only the preglycoprotein sequences. Both viral glycoproteins G2 and G1 could be synthesized and correctly processed in the absence of the 78- and 14-kDa proteins, as well as a large portion of the preglycoprotein sequences. However, the hydrophobic amino acid sequence immediately preceding the mature glycoprotein coding sequences was required for authentic glycoprotein production. The M-segment expression strategy involving aspects of translational initiation and protein processing are discussed. The functional roles of the 78- and 14-kDa proteins remain unclear.

Rift Valley fever is an important disease of both livestock and humans in much of sub-Saharan Africa, causing acute febrile disease, abortions, and death in cattle and sheep and a denguelike illness in humans. Rift Valley fever virus (RVFV), a member of the *Phlebovirus* genus of the *Bunyaviridae* family, is the etiologic agent of this disease. RVFV, like all viruses of the *Bunyaviridae* (1), possesses a tripartite RNA genome consisting of L, M, and S segments (11, 28, 32). The expression strategy of the genomic segments of members of this family of viruses has been under investigation. For expression of the S RNA, viruses of the *Bunyavirus* (La Crosse, snowshoe hare, Germiston, and Akabane) and *Uukuvirus* (Uukuniemi) genera use a "negative-sense" strategy in that viral-complementary subgenomic RNA species encode, in overlapping reading frames, the nucleocapsid protein (N) and a nonstructural protein (NS_S) (2, 3, 5, 6, 9, 15, 29, 30, 37). The S segment of Hantaan virus, the type species of the *Hantavirus* genus, also uses the negative-sense strategy, but apparently codes only for a nucleocapsid protein (34). Members of the *Phlebovirus* genus use a different means of S-segment expression. The N protein is expressed from a subgenomic viral-complementary mRNA, whereas the NS_S polypeptide is translated from a subgenomic viral-sense mRNA (17, 28). This expression strategy has been termed "ambisense" (17).

Expression of the M RNA segment of Bunyavirus, Uukuvirus, Hantavirus, and Phlebovirus members proceeds by the negative-sense strategy. Nucleotide sequence data indicate that the M segment RNA of these viruses possesses a single large open reading frame (ORF) in the viral-complementary RNA polarity that codes for both viral glycoproteins (11, 14, 18, 22, 33, 35).

Although viral-complementary RNA with a size similar to that of the L RNA has been observed (28, 30), direct studies of L-segment RNA expression have not been reported.

We have been interested in studying in greater detail the coding capacity and expression strategy of the RVFV M segment RNA. Our previous work (11) indicated that there are potential coding sequences in excess of that required for the two viral glycoproteins within the ORF of the M RNA of RVFV. The extent to which this additional information might be expressed as protein would depend on the selection of one of the five in-phase translation initiation codons that precede the mature glycoprotein coding sequences. To further investigate these issues, we generated site-specific antiserum reagents and constructed a series of recombinant vaccinia viruses into which defined sequences of the RVFV M segment were engineered. In this report we demonstrate the usefulness of these sera and viruses in identifying and characterizing the gene products and expression strategy of this *Phlebovirus* RNA segment.

MATERIALS AND METHODS

Viruses and cells. RVFV (strain ZH501 [11, 27]) was used to infect Vero E6 cell monolayers as previously described (11). All procedures involving infectious RVFV were conducted in the P3+ containment facilities at the Virology Division, U.S. Army Medical Research Institute for Infectious Diseases, Frederick, Md. Vaccinia viruses (wild-type WR strain and recombinant derivatives thereof) were propagated in BSC40 cells (12).

Immunologic reagents. Hyperimmune guinea pig antiserum to RVFV (αRV) and murine monoclonal antibodies (MAbs) to RVFV glycoproteins G2 (4-39-CC and 4-10-10A) and G1 (4G11 and 1-29-5F) were provided by J. Dalrymple, J. Meegan, and J. Smith of the U.S. Army Medical Research Institute for Infectious Diseases. Antiserum C12 was generated by immunization of mice with a bacterially produced G2-analog polypeptide comprising the amino-terminal 91%

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of the mature G2 glycoprotein and 59 amino acids of the RVFV ORF preceding these mature sequences (19). R895 and R900 are rabbit antisera generated against synthetic peptides corresponding to amino acids 16 to 30 (AVIRVSLS STREETC) and 40 to 51 (IEGAWDSLREEE), respectively, of the preglycoprotein region of the ORF. Peptides (supplied by Peninsula Laboratories, Inc., Belmont, Calif.) were conjugated to keyhole limpet hemocyanin by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (23) for peptide 16-30 or glutaraldehyde (31) for peptide 40-51. Rabbits were injected intradermally with 1 mg of conjugate emulsified in Freund complete adjuvant (primary injections) and boosted at 14-day intervals with conjugate in incomplete Freund adjuvant.

Radioimmunoprecipitation. BSC40 or Vero E6 cell monolayers were infected with vaccinia viruses (wild type or recombinants) at a multiplicity of infection of 5. At 2.5 h postinfection, the cells were incubated for 30 min in methionine-free medium and then for 2 h in methionine-free medium containing 100 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml. RVFV-infected Vero E6 cells (multiplicity of infection of 1) were similarly radiolabeled at 16 h postinfection. Infected, radiolabeled cells were harvested and disrupted in either detergent containing RIPA buffer (4) at 4°C or by boiling in a buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (SDS buffer [19]). All lysates were cleared by centrifugation at 90,000 × *g* for 30 min. Immunoprecipitations of aliquots of the cleared lysates were carried out as previously described (4). Samples of radiolabeled, immunoprecipitated proteins were electrophoresed in SDS-containing 10% or 10 to 15% gradient polyacrylamide gels (21), fluorographed (8), and exposed to X-ray film.

Endoglycosidase treatment. Washed immune complexes bound to Pansorbin (Calbiochem-Behring, La Jolla, Calif.) were eluted by boiling in 0.5% SDS–50 mM Tris hydrochloride (pH 7.2) for 4 min. After removal of the Pansorbin bacteria by centrifugation, the eluate was diluted 2.5-fold in 10 mM sodium phosphate buffer (pH 5.8)–1 mM phenylmethylsulfonyl fluoride. Endoglycosidases H (4 mU) and F (120 mU) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were added, and the mixtures were incubated at 37°C for 16 h.

Antigen quantitation. The relative amounts of RVFV glycoproteins present in cleared cell lysates were quantified by using an antigen-capture enzyme-linked immunosorbent assay (ELISA). Polystyrene plates (96 wells; Immulon-2; Dynatech Laboratories, Inc., Chantilly, Va.) were coated (at 4°C for 16 h) with a MAb (1:1,000 dilution of ascitic fluid in phosphate-buffered saline [PBS]) specific for either glycoprotein G2 (4-39-CC) or G1 (1-29-5F) and then blocked (at 37°C for 30 min) with PBS containing 2% bovine serum albumin. After the plates were washed three times with PBS containing 0.1% Tween 20, cleared RIPA lysates of virus-infected cells (normalized to 1.1 mg of total cell protein per ml based on the BCA assay [Pierce Chemical Co., Rockford, Ill.]) were serially diluted in PBS containing 0.1% Tween 20 and 2% bovine serum albumin and applied to duplicate wells of the MAb-coated plates. After incubation at 37°C for 2 h, the wells were washed three times as described above. Polyvalent anti-RVFV guinea pig serum (αRV; 1:1,000 dilution) was then added (37°C for 30 min). After the plates were washed as described above, horseradish peroxidase-conjugated goat immunoglobulin reactive against guinea pig immunoglobulins (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.; 1:1,000 dilution) was applied (37°C for 30 min.), the plates were washed again, and bound peroxidase-labeled antibody was measured by using 2-2′-azino-di-(3-ethyl-benzthiozoline sulfate) (ABTS; Kirkegaard and Perry). The indicator reaction was done at room temperature for 15 min, stopped with 1 M phosphoric acid, and read at 405 nm with a Dynatech Micro ELISA automated plate reader. For antigen quantitation, an RVFV-infected cell lysate dilution was arbitrarily assigned a unit value of 1. Serial dilution values for this lysate were plotted against ELISA reactivity ($\log_{10}$ dilution values versus $\log_{10}$ optical density at 405 nm). The resulting curve served as a standard for determination of the relative amounts of antigen present in recombinant vaccinia virus cell lysates by Lotus 1-2-3 linear regression analysis.

Construction of recombinant RVFV-vaccinia viruses. In general, all recombinant DNA procedures were performed as previously described (19, 25). The construction of plasmids pSCRV-7 and pSCRV-8 was carried out as follows. The insert of an RVFV cDNA clone (pRV51 [11]) containing the viral-complementary 5′-end region of the M segment was excised by *Pst*I restriction. To remove the 5′-end dG and dT homopolymeric tails (the result of cDNA cloning procedures) and to generate a library of progressive deletions at the 5′ end of the M segment, this fragment was treated with nuclease Bal 31 (New England BioLabs, Inc., Beverly, Mass.) as previously described (19), except that the concentration of sodium chloride in the buffer was reduced to 0.3 M. After *Nde*I restriction and DNA polymerase repair, deletion fragments in the size range of 450 to 570 base pairs were isolated and subcloned into the *Sma*I site of a plasmid having a *Bam*HI-*Sma*I-*Eco*RI polylinker. Plasmids from transformed *Escherichia coli* containing these 5′-end deletion inserts were prepared and initially screened for *Bam*HI (polylinker)-*Sst*I (RVFV nucleotide 183) fragment size by electrophoresis on polyacrylamide gels. Those with the desired-size fragments were then sequenced (26) to establish the 5′ endpoint of the deletion within the RVFV sequence. Two clones were selected for further manipulation. The 5′ endpoint of clone 7 was nucleotide 20, one nucleotide preceding the first translation initiation codon of the ORF (Fig. 1). The 5′ endpoint of clone 8 was nucleotide 132, three nucleotides before the second in-phase ATG codon (Fig. 1). These deletion fragments were excised by *Bam*HI digestion (polylinker *Bam*HI site and the unique RVFV *Bam*HI site at nucleotide 302) and inserted into *Bam*HI-restricted pRV-5. pRV-5 is a previously described derivative of the vaccinia virus transfer vector pGS20 (24) in which RVFV sequences from nucleotides 174 (at the third in-phase ATG codon) to 3874 were inserted into the pGS20 vector downstream of the vaccinia virus 7.5K promoter (10). The resultant plasmids possessed RVFV sequences from nucleotides 20 to 3874 (construct 7) and 132 to 3874 (construct 8). The entire RVFV insert was excised from each of these plasmids and inserted into the unique *Sma*I site in the vaccinia virus transfer vector pSC11 (7), yielding plasmids pSCRV-7 and pSCRV-8.

Construct pSCRV-6 was generated as follows. A DNA fragment comprising M segment sequences from the *Nco*I site (nucleotide 409) to nucleotide 3767 was excised from an intermediate vector (pSP76-6 [J. A. Suzich and M. S. Collett, submitted for publication]). After ends were blunted, this fragment was ligated into the *Sma*I site of pSC11. This plasmid (pSCRV-6) possesses M segment sequences beginning at the fourth in-phase ATG (nucleotide 411) and continuing through RVFV nucleotide 3767 (Fig. 1).

Plasmid pSCRV-9 was constructed in the following manner. The same intermediate vector used in the construction

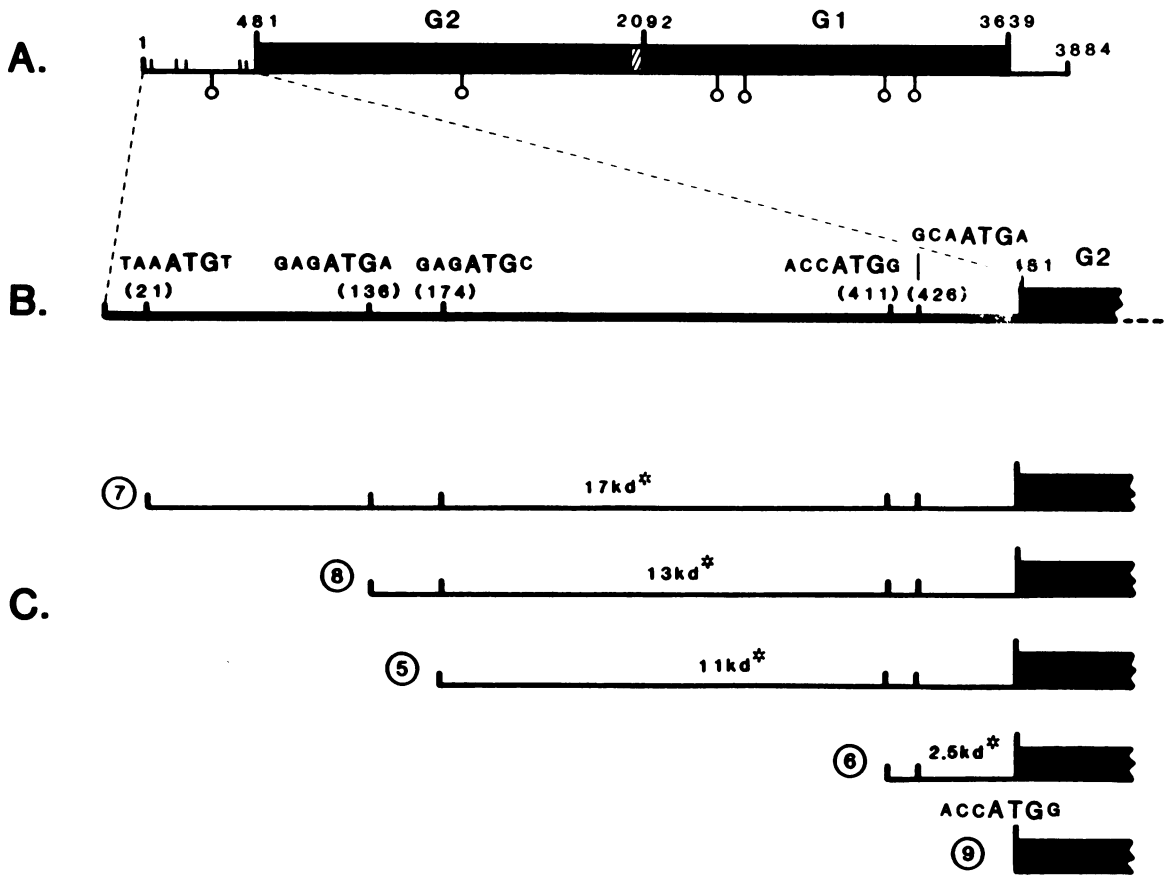

FIG. 1. RVFV M segment sequences present in recombinant vaccinia viruses. (A) Molecular organization of the RVFV M segment RNA. The numbers represent nucleotide coordinates of the M segment for the start of the mature glycoprotein coding sequences (nucleotides 481 to 2092 for G2 and G1, respectively) and the termination codon of the large ORF (nucleotide 3639). The closed bars indicate the glycoprotein-coding regions. The five in-phase ATGs preceding the start of the mature glycoprotein-coding sequences are denoted with vertical lines. Potential N-linked glycosylation sites (♁) are also shown. (B) Expanded representation of the preglycoprotein region showing the position and context of the five potential initiation codons. (C) Schematic representation of the 5′ M segment sequences present in the recombinant vaccinia viruses. The protein-coding potential (in kilodaltons [kd]) from the first available ATG to the beginning of the G2 coding sequences for each construct (*) is indicated.

of pSCRV-6 was linearized by *Nco*I restriction (RVFV nucleotide 409) and digested with nuclease Bal 31 as described previously (19). After *Sst*I restriction (RVFV nucleotide 1201), DNA deletion fragments approximately 720 base pairs long were isolated and reinserted into the original vector, which had been digested with *Nco*I and *Sst*I, the *Nco*I site having been made blunt by filling in the 5′ overhang with DNA polymerase and deoxynucleoside triphosphates. Deletion fragment inserts of plasmids from transformed *E. coli* were sized on a polyacrylamide gel. DNA sequencing of one isolate (clone 9) revealed a 5′ endpoint at RVFV nucleotide 483. In this clone, a *Nco*I site was regenerated, resulting in the replacement of the first codon for glycoprotein G2 (encoding glutamic acid) with a methionine codon (Fig. 1). The complete RVFV insert from clone 9 was excised from this intermediate plasmid by digestion at flanking restriction sites and ligated into the *Sma*I site of pSC11.

The pSC11-RVFV recombinant plasmids described above were linearized by *Sca*I restriction (unique site located in the β-lactamase gene) and transfected into vaccinia virus-infected BSC40 cells as described previously (24). Recombinant viruses, referred to hereafter as viruses 7, 8, 6, and 9, were isolated from blue plaques after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining (7), plaque purified twice, passed through a filter (pore size, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.), and plaque purified a third time before working virus stocks were prepared.

RESULTS

Coding capacity of the RVFV M segment. The RVFV M segment RNA possesses a single large ORF in the viral-complementary polarity with the capacity to encode 133 kilodaltons (kDa) of protein (11). After aligning the amino-terminal amino acid sequences of the two viral glycoproteins (G2 and G1) within this ORF, we previously indicated that two regions might potentially code for additional protein: a region from the first ATG codon of the ORF to the codon for the first amino acid of the mature G2 glycoprotein and a second "intragenic" area lying between the coding sequences for the two glycoproteins (Fig. 1A).

To address the question of the putative intragenic region, we attempted to generate antisera in rabbits to synthetic peptides corresponding to the coding sequences immediately preceding those for the second (G1) mature glycoprotein (Fig. 1A, hatched region). One peptide, representing the

amino acid sequence −12 to −1 with respect to the G1 coding sequences (LMLLLIVSYASA), failed to elicit antibodies to itself or any RVFV M segment polypeptide (data not shown). A second peptide, representing amino acids −27 to −16 upstream of glycoprotein G1 (encoded by nucleotides 2028 to 2054: APIPRHAPIPRY) was immunogenic. Radioimmunoprecipitation analyses demonstrated that antiserum to this peptide recognized glycoprotein G2 and a 78-kDa protein described below (data not shown). Therefore, the extent of any intragenic region between the G2 and G1 coding sequences must be minimal, if it exists at all. In fact, the sequences immediately preceding the G1 coding sequences encode quite hydrophobic amino acids (possibly explaining the insoluble and nonimmunogenic nature of the first peptide) and could represent a "leader" or signal sequence for the G1 glycoprotein. However, further work will be required to establish this.

From the first ATG of the ORF to the beginning of the coding sequences for glycoprotein G2 there is a potential coding capacity of 17 kDa (Fig. 1). To investigate protein expression within this preglycoprotein region, we generated antisera to synthetic peptides corresponding to amino acid sequences encoded between the first and second ATG (R895) and between the second and third ATG (R900). An immunoprecipitation analysis of a [^{35}S]methionine-labeled RVFV-infected cell lysate, using these antisera, is presented in Fig. 2. Antiserum R895 recognized a single polypeptide of 78 kDa, whereas R900 immunoprecipitated a 14-kDa protein, as well as the 78-kDa polypeptide. These two polypeptides represent previously uncharacterized gene products of the RVFV M segment. Also shown in Fig. 2 are the polypeptides

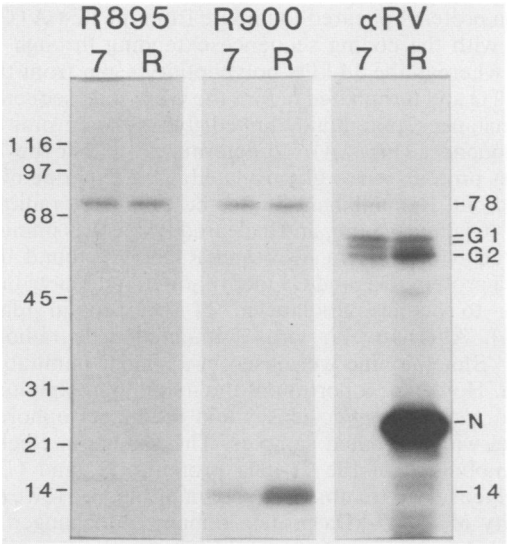

FIG. 2. RVFV M segment proteins expressed in RVFV-infected and recombinant virus 7-infected cells. Cleared lysates, solubilized by either SDS buffer or RIPA buffer, were prepared from [^{35}S] methionine-labeled recombinant 7-infected (lanes 7) and RVFV-infected (lanes R) Vero E6 cells. Portions of the SDS lysates were immunoprecipitated with rabbit antisera R895 and R900. The RIPA lysates were immunoprecipitated with serum from a RVFV-hyperimmunized guinea pig (αRV). Aliquots of the immunoprecipitates were electrophoresed on an SDS-containing 10 to 15% polyacrylamide gradient gel. The numbers at the left represent molecular masses (in kilodaltons) of standards. The notations in the right margin indicate the positions of the 78-kDa protein, glycoproteins G1 and G2, the viral nucleocapsid (N) protein, and the 14-kDa protein.

immunoprecipitated with an anti-RVFV serum (αRV): the M segment-encoded glycoproteins G2 and G1 and the 26-kDa nucleocapsid protein (N) encoded by the S segment RNA (28).

Expression of M-segment proteins in recombinant RVFV-vaccinia viruses. To further investigate these newly identified preglycoprotein gene products and the expression strategy of the RVFV M segment, we explored the potential usefulness of recombinant vaccinia viruses. We have previously shown that the RVFV glycoproteins G2 and G1 can be expressed in a recombinant RVFV M segment-vaccinia virus (10). However, this recombinant did not possess the complete ORF of the RVFV M segment. Therefore, we constructed recombinant virus 7. This virus contained the entire ORF of the M segment (Fig. 1) and expressed in virus-infected cells the full complement of M segment polypeptides found in authentic RVFV-infected cells: the 78-kDa protein, the 14-kDa protein, and both glycoproteins (Fig. 2). The fact that recombinant virus 7 accurately displayed the M segment expression pattern of authentic RVFV indicated that use of recombinant vaccinia viruses to study gene expression in this system was of value.

Immunoprecipitation using the preglycoprotein-region antipeptide serum (Fig. 2) not only identified the 78- and 14-kDa polypeptides but also provided information as to the sequences encoding these proteins. For example, the 78-kDa protein possessed sequences encoded between the first and second ATG, whereas the 14-kDa protein was unreactive with antibodies to this region. Since there are additional in-phase ATG codons between the first translation initiation codon and the glycoprotein gene (Fig. 1), these results raise several questions concerning the M segment expression strategy and features of translation initiation and protein processing. To investigate these issues, we exploited the recombinant vaccinia virus system and constructed a series of recombinant viruses in which the amino-terminal portion of the M segment ORF was truncated to various extents (Fig. 1C). Recombinants 8, 5, and 6 contain M segment sequences beginning just before the second, third, and fourth in-phase ATGs, respectively. Recombinant 9 lacks all preglycoprotein sequences and has a methionine codon (ATG) substituted for the first codon for the mature G2 protein (encoding glutamic acid). The RVFV sequence in all recombinants (including recombinant 7) then extends uninterrupted beyond the authentic termination codon of the M segment ORF (Fig. 1).

To identify RVFV-specific polypeptides in cells infected with these recombinant viruses, radiolabeled infected cell lysates were prepared from each and used in immunoprecipitation analyses in parallel with lysates from recombinant 7-infected cells. Initially, expression of glycoproteins G2 and G1 was investigated. Anti-RVFV serum (αRV) immunoprecipitated three closely migrating polypeptides (56 to 65 kDa) from cells infected with recombinants 7, 8, 5, and 6 (Fig. 3A). In virus 9-infected cells, only the upper two bands of this triplet were present. No additional polypeptides analogous to the 78-kDa protein found in cells infected with recombinant 7 were observed with any of the other viruses. Using a MAb to glycoprotein G2 (αG2), the band with the greatest mobility of the triplet (56 kDa) was immunoprecipitated from cells infected with viruses 7, 8, 5, and 6 (Fig. 2A). This MAb also reacted with the 78-kDa protein present in recombinant 7-infected-cell lysates. Immunoprecipitation of these cell lysates with a glycoprotein G1-specific MAb (αG1) showed that the 63- and 65-kDa polypeptides were present in all five recombinant virus-infected cells (Fig. 3A). In all of

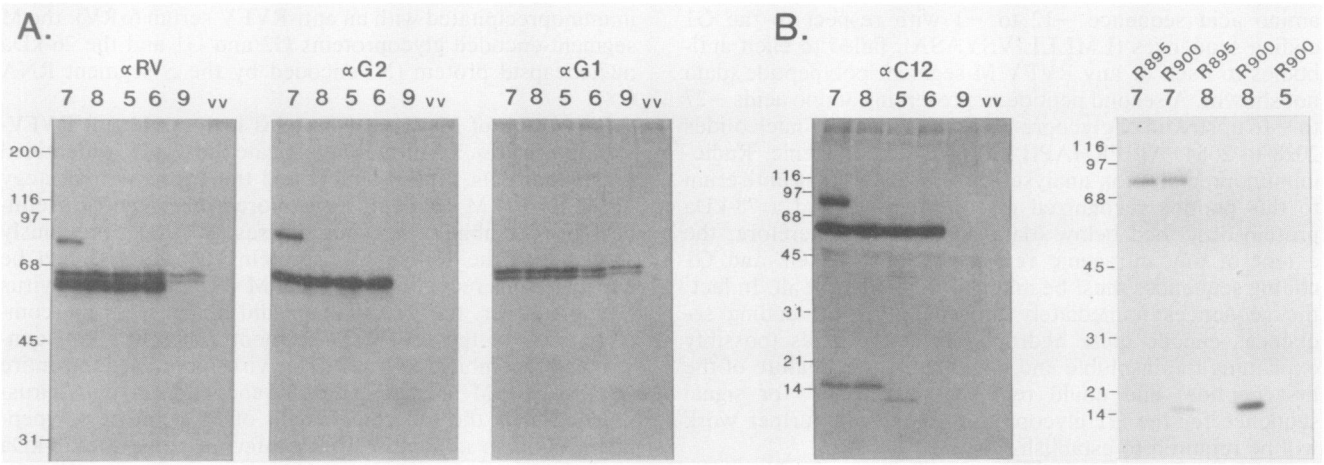

FIG. 3. RVFV-specific polypeptides expressed in recombinant vaccinia virus-infected cells. (A) Cleared RIPA lysates were prepared from [^{35}S]methionine-labeled wild-type (vv) or recombinant vaccinia virus-infected BSC40 cell cultures. Portions of each of these lysates were immunoprecipitated with either serum from a RVFV-hyperimmunized guinea pig (αRV), a MAb specific for glycoprotein G2 (αG2), or a MAb specific for glycoprotein G1 (αG1). Aliquots of the immunoprecipitates were electrophoresed on SDS-containing 10% polyacrylamide gels, which were subsequently fluorographed. The numbers above the lanes indicate the recombinant viruses used for infection. The positions of molecular size standards (sizes in kilodaltons) are indicated in the left margin. (B) Cleared lysates prepared in SDS buffer (see the text) from [^{35}S]methionine-labeled wild-type (vv) or recombinant virus-infected BSC40 cell cultures were used for immunoprecipitation with antisera C12 (αC12), R895, and R900 (see the text for description of antisera). Aliquots of the immunoprecipitates were electrophoresed on SDS-containing 10 to 15% polyacrylamide gradient gels, which were then fluorographed. Lanes and molecular sizes are indicated as in panel A.

the immunoprecipitation studies described above, a wild-type vaccinia virus-infected-cell lysate served as a negative control. Thus, all recombinant viruses, except virus 9, appeared to synthesize and correctly process both RVFV glycoproteins; virus 9 expressed only glycoprotein G1. Only recombinant 7 was able to produce the G2-specific 78-kDa protein, suggesting that synthesis of this polypeptide required the presence of the first ATG of the ORF.

To investigate protein expression within the preglycoprotein region of this series of recombinant vaccinia viruses, immunoprecipitations were carried out using several antisera specific to these sequences. C12 antiserum (αC12) was generated to a bacterially produced G2 analog containing the amino-terminal 91% of G2 and the 59 amino acids encoded by the sequence immediately preceding that for mature G2 (19). This antiserum immunoprecipitated the G2-specific 78-kDa protein (from recombinant 7-infected cells) and 56-kDa protein (from virus 7-, 8-, 5-, and 6-infected cells) (Fig. 3B). In addition, αC12 recognized the 14-kDa polypeptide expressed in recombinant 7-infected cells, the same 14-kDa protein in virus 8-infected cells, and a 12.5-kDa protein in virus 5-infected cells. Neither of these lower-molecular-weight proteins were present in virus 6-, virus 9-, or wild-type virus-infected-cell lysates (Fig. 3B).

As shown in Fig. 2, the antipeptide sera R895 and R900 both recognized the 78-kDa protein in recombinant 7-infected cells (Fig. 3B). No analogous polypeptides were immunoprecipitated with these sera from virus 8- or virus 5-infected cells. The 14-kDa protein, present in virus 7-infected cells was also immunoprecipitated from virus 8-infected cells by R900 antiserum but not by R895 antiserum, suggesting that the second ATG of the ORF may be used for the translational initiation of this protein. Neither of these antisera reacted with any polypeptides present in recombinant 5-infected cells (Fig. 3B). Therefore, the 12.5-kDa protein immunoprecipitated by αC12 in virus 5-infected-cell lysates may result from initiation at the third ATG and occur only when this ATG is the first available initiation codon of the ORF.

The 78- and 14-kDa proteins represent previously uncharacterized gene products of the RVFV M segment. The 78-kDa protein appeared to initiate from the first ATG of the ORF, with the coding sequence extending through the G2 gene, whereas the 14-kDa polypeptide began from the second ATG and terminated before the G2 coding sequences. In both instances, potential N-linked glycosylation sites exist in the sequences (Fig. 1A). To determine whether the 78- and 14-kDa proteins were glycosylated, two experiments were carried out. Recombinant 7-infected cells were radiolabeled with [^{14}C]glucosamine, and a cleared lysate was immunoprecipitated with antisera αRV and R900. We found that the 78-kDa protein, G2, and G1 incorporated radiolabel but were unable to detect radiolabeled 14-kDa protein (data not shown). Alternatively, virus 7-infected cells radiolabeled with [^{35}S]methionine were used in a similar immunoprecipitation. However, a portion of the immunoprecipitates were treated with endoglycosidases and then electrophoresed in parallel with untreated samples. The change in electrophoretic mobilities of the 78-kDa protein, G2, and G1 upon endoglycosidase treatment is shown in Fig. 4. However, the mobility of the 14-kDa protein remained unchanged. Thus, the 78-kDa protein, but not the 14-kDa protein, was glycosylated.

The data presented above indicate that the series of recombinant RVFV M segment-vaccinia viruses constructed in this study can serve as useful surrogate expression vectors for studying gene expression in this system. Because these viruses may have further utility in the future development of vaccines (10), it was of interest to compare the relative levels of RVFV glycoprotein production by cells infected with the recombinant vaccinia viruses and authentic RVFV. To do this, we used an antigen-capture ELISA (see Materials and Methods). The ELISA data were then used to calculate relative expression levels in the various virus-infected-cell

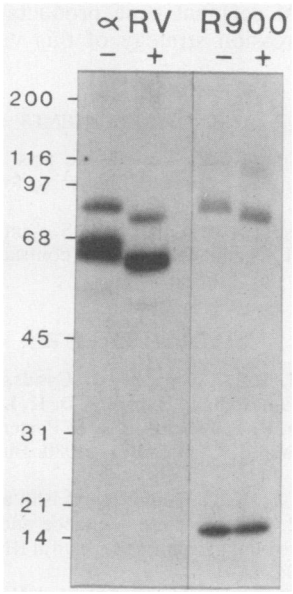

FIG. 4. Endoglycosidase treatment of M segment polypeptides. [^{35}S]methionine-labeled recombinant 7-infected BSC40 cells were solubilized by either RIPA buffer or SDS buffer lysis, and the cleared lysates were immunoprecipitated with serum from a RVFV-hyperimmunized guinea pig (αRV) or R900 antiserum, respectively. Washed immune complexes were eluted from Pansorbin, and portions of the eluates were treated (+) with endoglycosidases as described in the text. These portions were then electrophoresed in an SDS-containing 10 to 15% polyacrylamide gradient gel adjacent to similar portions of untreated (−) material. The numbers at the left indicate approximate molecular masses (in kilodaltons).

lysates (Table 1). Compared with authentic RVFV-infected cells, vaccinia virus recombinant 5- and 6-infected cells produced about half as much glycoprotein G2 and G1 (normalized for micrograms of total cell protein). Recombinant 7- and 8-infected cells yielded only a quarter as much of each glycoprotein. Virus 9-infected cells were severely deficient in glycoprotein G1 production and, as previously indicated, produced no glycoprotein G2.

DISCUSSION

Here we describe the use of specific antipeptide sera and the construction and use of recombinant vaccinia viruses to study the coding capacity and expression of the M genomic segment of a phlebovirus. We found that a recombinant virus possessing the entire M segment ORF of RVFV (virus 7) produced the same constellation of M segment-encoded proteins in infected cells as that found in authentic RVFV-infected cells. The M segment could be completely and faithfully expressed in the vaccinia virus system, the gene products apparently being correctly processed and modified, in the absence of the L and S genomic segments and their products. Thus, the use of recombinant vaccinia viruses as a model system to further explore *Phlebovirus* expression and gene product function is indicated.

The availability of such a faithful model system offers particular advantages for the study of RVFV in that it reduces the need for direct manipulation of an exotic pathogen. In the absence of infectious RVFV, we may safely carry out detailed biochemical and genetic manipulations to investigate features of viral replication and gene function, as well as explore new avenues for vaccine development (10).

The constellation of proteins found to be encoded by the M segment included, in addition to the viral glycoproteins G2 and G1, two previously uncharacterized polypeptides: a glycosylated 78-kDa protein and a nonglycosylated 14-kDa polypeptide. Previously, Rice et al. (32) described a 100-kDa protein and Struthers et al. (36) described an 80-kDa protein, both specific to RVFV-infected cells. Although in both studies the nature of these proteins was unclear, they might represent the 78-kDa M segment product described here.

With respect to the RVFV 14-kDa preglycoprotein polypeptide described herein, other investigators have made use of genetic reassortants among closely related bunyaviruses to identify M segment-encoded, apparently nonstructural proteins of similar molecular mass (13, 16). Based on the gene organization predicted from the nucleotide sequences of the M segments of viruses used in these reassortant studies (14, 22), these putative "NS_m" proteins are probably encoded by preglycoprotein sequences present in their ORFs, although this has not been demonstrated directly. Another phlebovirus, Punta Toro virus, also possesses preglycoprotein coding sequences, in this instance with a coding potential of up to 30 kDa (18). Identification of a polypeptide product from these sequences has not been reported. As for members of other genera of the *Bunyaviridae*, representatives of the *Hantavirus* and *Uukuvirus* genera appear to lack sequences in their M segments capable of encoding such NS_m polypeptides (33, 35).

The use of site-specific antibody reagents and manipulation of the RVFV sequences present in the recombinant vaccinia viruses revealed several intriguing features of RVFV M segment expression. Since the M segment possesses a single ORF yet yields four polypeptide products, clearly mechanisms of proteolytic processing are involved in the biogenesis of these proteins. Indeed, cell-free translation studies of RVFV M segment mRNA-like transcripts demonstrated that the primary translation product, which has never been observed in virus-infected cells, was a polyprotein of about 133 kDa encompassing the entire ORF (Suzich and Collett, submitted). This protein was cotranslationally processed in the presence of microsomes to yield the 78- and 14-kDa proteins and glycoproteins G2 and G1 observed in this study in RVFV- and recombinant vaccinia virus-infected

TABLE 1. RVFV glycoprotein expression in recombinant vaccinia virus-infected cells

Virus	Relative expression level[a]		Virus titer[b]
	G2	G1	
RVFV	1.0	1.0	8.5×10^7
7	0.24	0.24	2.0×10^8
8	0.28	0.22	2.0×10^8
5	0.49	0.49	2.5×10^8
6	0.51	0.44	1.5×10^8
9	0.00	0.01	1.0×10^8

[a] Antigen-capture ELISA data were used to calculate the relative levels of glycoproteins G2 and G1 as follows. The RVFV-infected-cell lysate was arbitrarily assigned a value of 1. Serial dilutions of this lysate and their ELISA reactivity were used to construct a standard curve for calculation of the relative amount of protein present in recombinant virus-infected cell lysates. Each entry in the table for the recombinant viruses represents an average of unit values obtained at two dilutions (four determinations per dilution) within the linear portion of the antigen reactivity curve.

[b] Virus titers were determined by plaque assay at the time of cell harvest for lysate preparation (16 h postinfection). Cell-free culture fluid was used to determine the titer of RVFV, while the titers of the recombinant vaccinia viruses were determined with infected cells that had been disrupted by freeze-thaw and sonication.

cells. However, in addition to proteolytic processing, based on the data presented in this report, it further appears that differential translation initiation could also be involved in M segment gene expression. Two different initiation codons within the same ORF may be used to direct the synthesis of two distinct polypeptide products with partially identical overlapping sequences. The 78-kDa protein initiated at the first ATG of the ORF and encompassed the entire preglycoprotein and glycoprotein G2 coding sequences. The 14-kDa protein began at the second in-phase ATG and was composed of only preglycoprotein sequences. Whether the 14-kDa protein represents the product of an independent translational initiation or arises as a proteolytic processing product of some unidentified precursor is not clear. The fact that virus 8, which initiates translation at the second ATG, yields a 14-kDa protein with an identical electrophoretic mobility seems to favor, but not prove, the former alternative.

These considerations also raise questions concerning the mechanism for generation of the viral glycoproteins. The actual translational initiation site which ultimately yields the mature glycoproteins G2 and G1 was not addressed in the present work. The recombinant viruses studied here did show that mature glycoproteins could be synthesized after the elimination of each of the first three ATG codons in the preglycoprotein region. However, this has little bearing on the translational initiation event in the natural situation (with all five ATGs of the preglycoprotein region present) required to give rise to a putative precursor of mature glycoproteins G2 and G1. Pulse-chase experiments failed to convincingly demonstrate that the 78-kDa protein serves as a precursor to glycoprotein G2 or the 14-kDa protein. It is as likely that glycoprotein G2 or the 14-kDa protein are efficiently processed products of a precursor initiated at the second ATG codon. Work is in progress to distinguish among these alternatives by construction of site-specific mutants lacking select ATG codons.

Recombinant virus 6, which possesses RVFV M segment sequences beginning at the fourth in-phase ATG, encoding only 22 amino acids before the glycoprotein sequences, efficiently produced mature G2 and G1. Apparently, the 78- and 14-kDa proteins and a large portion of the preglycoprotein sequence are not functionally required for, or involved in, correct glycoprotein expression and processing. However, removal of the 22 amino acids preceding the mature glycoprotein G2 and/or change of the first amino acid of G2 from glutamic acid to methionine (recombinant 9) resulted in a dramatic change in glycoprotein expression. No glycoprotein G2 was detectable, whereas glycoprotein G1 appeared correctly processed and modified, albeit at significantly reduced levels. The initiation codon engineered into virus 9 is in an optimized context (20) and is in fact the same sequence used in virus 6. Cell-free translation of an RNA transcript analogous to the M segment sequences of virus 9 demonstrated that this initiation codon was used very efficiently in vitro (Suzich and Collett, submitted). Thus, it seems unlikely that the absence of glycoprotein G2 in virus 9-infected cells was due to inefficient translation initiation. The 22 amino acids removed to generate virus 9 are quite hydrophobic (11) and may represent a leader or membrane insertion signal necessary for proper glycoprotein processing or stabilization. The means by which glycoprotein G1 was expressed in the complete absence of G2 in recombinant 9 remains a matter for speculation. Further investigations to identify the actual translation initiation events and intermediate processing products involved in the biogenesis of each of the RVFV M segment gene products will be required before the expression strategy of this viral RNA will be understood.

ACKNOWLEDGMENTS

We thank Joanne Kucks for graphic arts, Sherri Pierson for manuscript preparation, and Joel Dalrymple for many helpful discussions.

This work was supported in part by U.S. Army Medical Research and Development Command research contract DAMD 17-85-C-5226.

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