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RIFT VALLEY FEVER VIRUS: MOLECULAR BIOLOGIC STUDIES OF THE M SEGMENT RNA FOR APPLICATION IN DISEASE PREVENTION

ANNUAL REPORT

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The molecular and biologic properties of the M segment RNA of Rift Valley fever virus (RVFV) were studied, with focus on elucidation of the expression strategy of this molecule. The results indicate the expression strategy of the RVFV M segment is surprisingly complex. Expression of the full complement of M segment proteins involves multiple translational initiation events giving rise to primary translation products which are co-translationally processed to yield the mature proteins. The first ATG codon of the single ORF is required for production of the 78kd protein. Independent translational initiation at the second in-phase ATG codon yields the 14kd protein. Biogenesis of glycoprotein G2 is dependent on translation start sites within the preglycoprotein region, but does not involve use of the first ATG codon of the ORF. Production of glycoprotein G1 appears to be largely independent of all ATG codons that precede the mature glycoprotein coding sequences. The two-site translational initiation strategy employed for the expression of the 78kd and 14kd proteins serves to control glycosylation and proteolytic processing of the resultant polypeptides. Recombinant RVFV-vaccinia viruses were used to define protein determinants responsible to eliciting protective immunity in animals. The importance of the carboxy-terminal portion of glycoprotein G2 was demonstrated.

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SUMMARY

The purpose of this work is to elucidate the molecular and biologic properties of the M segment RNA of the phlebovirus Rift Valley fever virus (RVFV). Work during this final year of this contract concentrated on investigations of the expression strategy of this molecule. We have employed cell-free transcription-translation systems, recombinant vaccinia viruses, sequence-specific immunologic reagents, and site-directed mutagenesis techniques to address this issue. Our results indicate the expression strategy of the RVFV M segment is surprisingly complex. The single large open reading frame (ORF) of the M segment mRNA encodes four gene products: the two viral glycoproteins G2 and G1, a glycosylated 78kd protein, and a nonglycosylated 14kd protein. The expression of the full complement of M segment-encoded proteins involves multiple translational initiation events giving rise to primary translation products which are co-translationally processed to yield the mature proteins. The first ATG codon of the M segment ORF is required for the production of the 78kd protein. Independent translational initiation at the second in-phase ATG codon yields the 14kd protein. The means by which the two envelope glycoproteins are generated is less clear. The biogenesis of glycoprotein G2 is dependent on translation start sites within the preglycoprotein region, but does not involve use of the first ATG codon of the ORF. The production of glycoprotein G1, on the other hand, appears to be largely independent of all ATG codons that precede the mature glycoprotein coding sequences. It is clear that the biogenesis of the two envelope glycoproteins of RVFV proceeds along two distinctly different pathways. The two-site translational initiation strategy employed for the expression of the 78kd and 14kd proteins serves to control post-translation protein modifications (glycosylation and proteolytic processing) of the resultant polypeptides. The recombinant RVFV-vaccinia viruses were further exploited for the definition of protein determinants responsible to eliciting protective immunity in animals. Glycoprotein G2 was previously shown to be a sufficient protective immunogen. Here, the importance of the carboxy-terminal portion of glycoprotein G2 was demonstrated.

FOREWORD

In performing the recombinant DNA experiments described in this report, the investigators have abided by the National Institutes Of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

In conducting laboratory animal immunization experiments described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publilcation N.(NIH) 78-23, Revised 1978).

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A. Introduction. This report summarizes work performed during the third and final year of this contract. The mission of this contract has been to elucidate the molecular and biologic properties of the middle (M) genomic segment of Rift Valley fever virus (RVFV), while keeping an eye out for applications of the basic research information toward strategies for disease prevention. We have investigated the coding capacity and expression strategy of this genomic segment employing cell-free transcription-translation systems and recombinant vaccinia vruses. We have found the M segment encodes primary translation product(s) of about 133kd (kilodaltons) representing the entire open reading frame (ORF), which appears to be co-translationally processed in virus-infected cells (1). The processed products are the viral glycoproteins G2 and G1, and two newly characterized polypeptides: a glycosylated 78kd protein and a non-glycosylated 14kd protein (2). The 78kd protein initiates at the first ATG of the ORF and encompasses the entire preglycoprotein and glycoprotein G2 coding sequences. The sequence of the 14kd protein begins from the second in-phase ATG and represent only preglycoprotein sequences. All of these M segment products are largely Golgi-localized in both RVFV-infected and recombinant vaccinia virus-infected cells (3). The 78kd and 14kd proteins, and most of the preglycoprotein sequence are not required for the proper synthesis, processing, modification, or Golgi localization of the viral envelope glycoproteins (2,3). However, the 22 amino acids preceding the glycoprotein coding sequences are important. Deletion of this sequence resulted in abolition of G2 expression, a dramatic decrease in G1 production, and the appearance of G1 at the cell surface (2, unpublished data). The utility of recombinant vaccinia viruses in the study of phlebovirus proteins and gene expression has been clearly demonstrated. The recombinant RVFV-vaccinia viruses are also viable live virus vaccine candidates. Mice immunized with a recombinant virus expressing glycoproteins G2 and G1 developed high virus neutralizing antibody titers and were completely protected against lethal RVFV challenge (4).

In this third year of study, we have further exploited the recombinant vaccinia virus system, as well as cell-free transcription-translation and site-directed mutagenesis techniques, to gain additional insights into the molecular and biologic properties of the RVFV M segment.

B. RVFV M Segment Expression Strategy. Members of Bunyaviridae appear to employ two fundamentally distinct

strategies for gene expression: "negative sense" and "ambisense". The M segment of RVFV employs a negative sense strategy (5,6). Within the M segment mRNA there exists a single ORF from which four distinct polypeptide products are generated: viral envelope glycoproteins G2 and G1, a 78kd protein, and a 14kd protein. Elucidating the mechanisms involved in the biogenesis of these proteins has been a major objective of our work.

1. Biogenesis of the 78kd and 14kd proteins. The fact that the M segment mRNA consists of a single ORF suggests biogenesis of the mature proteins proceeds by proteolytic processing of a primary translation product. Indeed, the cell-free translation of M segment mRNA-like transcripts revealed a primary polyprotein product encompassing the entire ORF which was co-translationally processed *in vitro* in the presence of microsomes to yield mature proteins (1). However, on occasion, we were able to resolve what appeared to be multiple, closely-spaced primary translation products in this cell-free system. This led us to consider that multiple translational initiation events within the same ORF might also be involved in the biogenesis of M segment proteins; there are four additional in-phase ATG codons downstream of the first ATG within the preglycoprotein region of the ORF (Fig.1). In fact, based on sequence-specific antibody recognition, the 14kd protein neatly lacked reactivity with antisera directed at sequences between the first and second ATG codons of the ORF, but did react with antibodies to the 12 amino acid sequence between the second and third ATGs; the 78kd protein reacted with immune reagents for both regions (1,2). Thus, there are two possible mechanisms for the biogenesis of the 14kd protein: this polypeptide arises as a proteolytic processing product of the 78kd protein or it represents the product of an independent translational initiation event at the second in-phase ATG codon of the ORF.

In an initial effort to investigate these two alternatives, we performed a pulse-chase experiment. Cells infected with a recombinant vaccinia virus which correctly expresses, processes, and transports all M segment proteins (virus 7; references 2,3) were pulse-labeled with ^{35}S-methionine and subsequently chased for various periods of time in the presence of a large excess of unlabeled methionine (Fig.2). This type of experiment, performed under several conditions (different pulse times; chase in the presence or absence of cycloheximide), failed to suggest a precursor-product relationship between the 78kd and 14kd proteins.

To directly assess the roles of the first and second in-phase ATG codons of the ORF on protein expression, recombinant vaccinia viruses were constructed in which one or the other of these codons was missing (see Fig.3). The M segment sequences in virus 8 (previously described in reference 2) begin just before the second ATG codon; this

virus lacks the first methionine codon, as well as the next 36 amino acids of the M segment ORF. Virus 8 failed to produce any 78kd protein, while production of the 14kd protein and glycoproteins G2 and G1 was unaffected (Fig.4A). Another recombinant virus, virus 72, is identical to virus 7 with the exception that the second in-phase ATG codon has been changed to CTC, therefore encoding leucine instead of methionine. Virus 72 produced normally the 78kd protein and glycoproteins G2 and G1, but yielded absolutely no 14kd protein (Fig.4B). Thus, the first ATG codon was necessary for synthesis of the 78kd protein, but not for the 14kd protein, and the second ATG was essential for 14kd protein production, but not for 78kd protein synthesis.

These data show the M segment of RVFV employs two different in-phase translational initiation codons to produce two distinct polypeptide products, the 78kd and 14kd proteins. Cell-free translation studies indicated the primary product of M segment mRNA translation to be protein species the size expected of a polypeptide encompassing the entire ORF (1). Taken together, these data would predict there to be two primary translation products from the same M segment mRNA: one inititating from the first ATG codon and extending to the termination codon of the ORF (1197 amino acids) and a second beginning from the second in-phase ATG and continuing to the end of the ORF (1159 amino acids). Using sequence-specific antiserum reagents and gel analyses of sufficient resolution, we have recently been able to clearly resolve these two primary translation products (unpublished data). Thus, biogenesis of the complete complement of M segment-encoded proteins involves both multiple translational initiation events and co-translational proteolytic processing of precursor polyproteins.

2. Biogenesis of glycoproteins G2 and G1. Although the above data appear to explain the mode of production of the 78kd and 14kd proteins, they do not address directly the biogenesis of the viral glycoproteins G2 and G1. As these proteins were produced normally by both recombinant virus 8 and 72, it appears neither the first nor second in-phase methionine codons are absolutely required for the generation of glycoproteins G2 and G1. Moreover, from pulse-chase experiments (Fig. 2), there is no indication the 78kd protein, inititated from the first ATG, serves as a precursor to glycoprotein G2.

These observations suggest alternative options exist for the biogenesis of glycoproteins G2 and G1. As there exist three additional in-phase ATG codons preceding the mature glycoprotein coding sequences, it was possible these may be involved in the synthesis of G2 and G1. To investigate this possibility, we constructed a series of "ATG mutant" recombinant vaccinia viruses analogous to virus 72, each possessing the first ATG of the ORF but having had mutagenized additional ATG codons within the preglycoprotein region. Thus, in virus 723, the second and third methionine

codons were changed to leucine and isoleucine, respectively. Virus 7234 additionally had the fourth in-phase methionine codon changed to isoleucine. And finally, virus 72345 completed the series with the mutation of the fifth methionine codon to that of isoleucine added to the preceding changes, leaving in this virus the first ATG of the ORF as the only translation start site before the glycoprotein coding region. These recombinant viruses are schematically summarized in Fig. 3.

Analyses of RVFV M segment proteins produced by cells infected with these viruses is shown in Fig. 5. All viruses directed the synthesis of comparable levels of the 78kd protein. The progressive removal of the internal translation start sites, however, had a profound negative affect on the biosynthesis of glycoprotein G2. In contrast, production of glycoprotein G1 appeared to be only minimally affected by the removal the these same methionine codons. These results are presented in a more quantitative fashion in Fig. 6, in which the relative amounts of each glycoprotein have been compared to that of the 78kd protein from each virus. With the progressive removal of internal methionine codons, there was a progressive reduction in the synthesis of glycoprotein G2, reaching a level of only 20% of wild-type in virus 72345. Glycoprotein G1 synthesis, on the other hand, was maintained at about 83-94% the level of wild-type production. It can be concluded from these analyses that efficient biosynthesis of glycoprotein G2, but not glycoprotein G1, is dependent on translation start sites within the preglycoprotein region other than the first ATG of the ORF.

The fact that virus 72345 still produced near wild-type levels of glycoprotein G1 might implicate the first ATG of the ORF as being responsible for this protein's production. To directly examine this, we constructed another recombinant vaccinia virus. Virus 10 possessed sequences of the M segment ORF beginning at amino acid 51 (just before the third ATG of the ORF) and continuing to the authentic termination codon. It also possessed the mutational changes at the third, fourth, and fifth in-phase methionine codons. Therefore, virus 10 lacked all ATG codons preceding the mature glycoprotein coding sequences. As virus 10 lacked the first ATG codon of the ORF, as expected, no 78kd protein was produced in virus 10-infected cells (data not shown). Surprisingly, however, the ability of virus 10 to direct the synthesis of glycoproteins G2 and G1 was similar to that of virus 72345; that is, a small amount of glycoprotein G2 (15-20% of wild-type) and near normal levels (75-80% of wild-type) of glycoprotein G1 (data not shown). These results add further complexity in our attempt to understand the expressior strategy of this genomic segment. They suggest not only are internal ATG codons important to protein biogenesis, but non-ATG translation start sites could also be involved. In any event, they serve to demonstrate that the biogenesis of the two major envelope glycoproteins of RVFV proceeds along two distinctly different pathways.

C. RVFV M Segment Protein Glycosylation. Within the M segment ORF, there exist six sites with the sequence Asn-X-Thr/Ser for possible N-linked glycosylation: one present in the preglycoprotein region within the coding sequences of both the 78kd and 14kd proteins; one site in the glycoprotein G2 coding region, also present in the 78kd protein; and four sites within glycoprotein G1 (Fig. 1). Analyses of the G2 and G1 glycoproteins from RVFV-infected cells have indicated that all of their potential sites for N-linked glycosylation are probably occupied with oligosaccharides (Alonso-Caplan and Dalrymple, personal communication). Concerning the remaining two M segment proteins, we had previously shown the 78kd protein was glycosylated and the 14kd protein was not (2). Thus, at least in the 14kd protein, the preglycoprotein glycosylation site is not utilized. However, in those studies, we did not determine whether one or both sites present in the 78kd protein were occupied with oligosaccharide.

To investigate the number of glycosylation sites utilized in the 78kd protein, as well as glycoproteins G2 and G1, we employed recombinant vaccinia virus technology to express M segment gene products and enzymatic deglycosylation to progressively remove glycans from each protein. The individual RVFV glycoproteins were immunoprecipitated with specific antisera from ^{35}S-methionine-labeled cells infected with either recombinant vaccinia virus 7 or recombinant virus 6. These viruses possess either the entire RVFV M segment ORF (virus 7) or only the coding domains of glycoproteins G2 and G1 (virus 6) (see reference 2). An aliquot of the washed, immunoprecipitated proteins was removed, and the remainder was treated for various times with endoglycosidase F. Portions of the untreated and endoglycosidase-treated samples were then electrophoresed in SDS-containing polyacrylamide gels. Over the time course of endoglycosidase treatment of the 78kd protein, two new and distinct polypeptide species were seen, representing obvious shifts in electrophoretic mobility from the untreated sample (Fig. 7). Similar analysis of glycoprotein G2 showed a single shift to a more rapidly migrating polypeptide. The situation with glycoprotein G1 was more complicated. Glycoprotein G1 exists in cells (whether recombinant vaccinia virus- or RVFV-infected) as a closely spaced doublet (2,4). The nature of the differences between the two molecular species of G1 remains unclear. In any event, endoglycosidase treatment of the doublet gave rise to a pattern of mobility shifts that is difficult to decipher. However, we feel we can reliably distinguish at least three distinct species generated during the course of deglycosylation.

The results of these endoglycosidase mobility shift experiments suggest glycoprotein G2 is glycosylated at one site and glycoprotein G1 at at least three sites. Certainly, the data for glycoprotein G2 was as expected; this protein

has only a single site for N-linked glycosylation. For glycoprotein G1, additional work will be necessary to more firmly establish its glycosylation status. However, the most interesting result from these experiments pertains to the 78kd protein. The data suggest that both sites for N-linked glycosylation in the 78kd protein were occupied with glycan. This finding is significant in that the glycosylation site within the preglycoprotein region of the ORF is also present in the 14kd protein but is not utilized.

To further examine glycosylation at the preglycoprotein region site, we constructed a series of recombinant vaccinia viruses in which the front-end (amino terminus) of the ORF remained intact, but which possessed truncations to various extents at the carboxy-end. Specifically, three viruses were made (Fig. 8). In virus 7-1539, the ORF was terminated at nucleotide 1539. This virus possesses both glycosylation sites present in the 78kDa protein. In viruses 7-1092 and 7-888, the ORFs were terminated at nucleotides 1092 and 888, respectively. The resultant RVFV ORFs in both viruses possess only a single site for N-linked glycosylation, that site in the preglycoprotein region (Fig. 8).

RVFV protein expression in cells infected with these recombinant viruses was characterized using three sequence-specific antiserum reagents. Antiserum R970 is directed against sequences between the first and second ATG codons of the RVFV ORF (1), and therefore is able to detect polypeptides that initiate from the first ATG codon ("78kd-like" proteins). As is shown in Figure 9, first panel, this antiserum specifically immunoprecipitated a single polypeptide from lysates of each of the three virus-infected cells which was of the approximate size expected for a protein encompassing the entire RVFV ORF present in each virus. The bands at 45kd and 17kd in the fluorograph of the R970 immunoprecipitation represent non-specific polypeptides recognized by this particular rabbit serum. Antiserum R900 is specific for the amino acid sequence between the second and third ATG codons of the RVFV ORF (2). In addition to the respective 78kd-like polypeptides, this antiserum identified the 14kd protein, initiating from the second ATG codon, produced by each of the three viruses (Fig. 9, second panel). Finally, an antiserum specific for glycoprotein G2 sequences was used to immunoprecipitate radiolabeled lysates of cells infected with each virus. Like the above antisera, the G2 antiserum also immunoprecipitated the 78kd-like polypeptides. In addition, a protein ("G2-like" protein) which approximated in size the coding capacity between the start of the mature G2 sequence to the end of the respective ORF was immunoprecipitated in each case (Fig. 9, third panel). As expected, the 14kd protein was not recognized by the G2 antiserum.

To determine whether the various polypeptides produced by these three viruses were glycosylated, ^{35}S-methionine-labeled, immunoprecipitated proteins were treated with

endoglycosidase F under conditions that removed all attached glycans. Immunoprecipitations were carried out with antisera R900 and G2 combined so all RVFV polypeptides produced by each virus could be analyzed together. Portions of the endoglycosidase-treated materials were electrophoresed adjacent to untreated samples in an SDS-containing polyacrylamide gel (Fig. 9, fourth panel). The 78kd-like proteins produced by each virus showed a mobility shift upon endoglycosidase treatment, indicating their possession of N-linked glycan. Since there is only one glycosylation site in the RVFV sequences in viruses 7-888 and 7-1092, these data demonstrate the utilization of the preglycoprotein region glycosylation site in the 78kd-like polypeptides initiated from the first ATG codon. However, in all cases, the 14kd protein initiated from the second in-phase ATG codon showed no mobility shift with endoglycosidase treatment, and therefore no evidence of glycosylation (Fig. 9, fourth panel). The G2-like polypeptides produced by virus 7-888 (poorly visible in the figure) and virus 7-1092 lack glycosylation sites and showed no change in migration when incubated with endoglycosidase. In virus 7-1539, the G2-like protein did shift, indicating utilization of the glycosylation site in the G2 protein coding sequence (Fig. 9, fourth panel).

The above data indicate that when proteins are initiated from the first ATG codon of the RVFV ORF, the preglycoprotein region glycosylation site is used for glycan attachment. On the other hand, translation initiation from the second ATG codon for the synthesis of the 14kd protein results in the failure of this same glycosylation site to be used. The results also suggest the glycosylated 78kd-like polypeptides (and the 78kd protein itself) were not proteolytically cleaved at the preglycoprotein region-glycoprotein G2 junction. This has been confirmed by pulse-chase experiments (Fig. 2). This is in contrast to the unglycosylated 14kd proteins, which in all cases were released by apparent cleavage at this site. These observations suggest the possibility that glycosylation at the preglycoprotein region site, apparently determined by site of translation initiation, might in turn influence (block) proteolytic processing at the preglycoprotein-glycoprotein G2 junction.

To investigate this, we constructed a recombinant vaccinia virus in which this glycosylation site was altered so as not to be a glycan attachment site. The RVFV sequences in virus 7N1 are identical in every respect to virus 7 with the exception that the preglycoprotein region glycosylation site Asn-Ile-Thr was changed to Ser-Ile-Thr by site-directed mutagenesis techniques. Analysis of RVFV proteins synthesized in virus 7N1-infected cells, compared to those in virus 7-infected cells, showed normal expression levels of all four M segment gene products (Fig. 10). The only difference noted between the RVFV proteins produced by the two viruses was the slightly more rapid electrophoretic

mobility of the 78kd protein produced by virus 7N1. This migration difference can be accounted for by the lack of carbohydrate at the preglycoprotein glycosylation site. The 7N1 virus 78kd protein was, however, glycosylated at the site within the G2 coding sequences (data not shown). Thus, elimination of the preglycoprotein region glycosylation site, and therefore prevention of glycosylation at this site in the 78kd protein did not alter the pattern of proteolytic processing of M segment polypeptides. The data relating to virus 7N1 do, however, confirm the utilization of both N-linked glycosylation sites in the 78kd protein.

D. Mapping RVFV Protein Determinants Required for Animal Protection. Using recombinant vaccinia viruses expressing individual RVFV M segment proteins, we previously showed glycoprotein G2 is a sufficient protective immunogen (see Annual Report for this contract dated August, 1987). To further define and localize the antigenic determinants of glycoprotein G2 capable of inducing protection in mice, we evaluated the series of recombinant viruses possessing the glycoprotein G2 COOH-terminal truncations. In addition to those described in Fig. 8 (viruses 7-888, 7-1092, 7-1539), two additional viruses were included: viruses 7-1860 and 7-2097; the ORFs terminating at nucleotides 1860 and 2097, respectively. Virus 7-2097 expresses the complete coding sequence of glycoprotein G2. In work done by J.M. Dalrymple and S.E. Hasty of USAMRIID, outbred and inbred mice were immunized by tail scarification. Four weeks following vaccination, mice were challenged with 1000 LD_{50}s of virulent RVFV. As is shown in Fig. 11, immunization with either virus 7-2097 or virus 7-1860 was capable of protecting all the animals. However, viruses expressing glycoprotein G2 analogues with more extensive COOH-end truncations offered little or no protection. These results are provocative in view of previously mapped antigenic determinants reactive with protective monoclonal antibodies (7). Of the two domains mapped, both are resident within the sequences expressed by virus 7-1539, one within those of virus 7-1092, and only a portion of one within those of virus 7-888. Possible interpretations of these data include: (i) the presence of important protective epitopes near the carboxy terminus of G2, (ii) the requirement for a complete COOH-terminus of G2 for proper cellular processing of antigenic viral components, or (iii) the carboxy terminus of glycoprotein G2 is important for the presentation and/or conformation of epitopes located within the amino proximal portion of the molecule.

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FIGURE 1

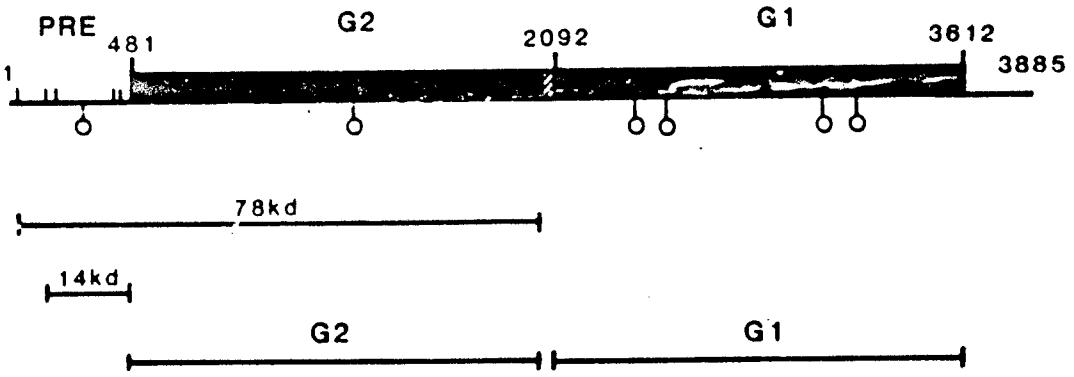

Figure 1. Schematic representation of the RVFV M segment RNA and its protein products. The RNA is presented in the viral-complementary polarity. Nucleotide coordinates for the beginning of the mature G2 (481) and G1 (2092) glycoproteins, the termination codon for the ORF (3612), and the end of the genome (3885) are given. The latter two numbers represent corrected values, as an error was found in the original sequence; an adenine has been added at nucleotide position 3494 (8). The vertical tick marks within the preglycoprotein region (PRE) represent the five in-phase ATG codons preceding the glycoprotein coding sequences. The lollipop figures depict the positions of the N-linked glycosylation recognition sequence: Asn-X-Thr/Ser. The four gene products and the regions within the ORF of their coding sequences are shown below the M segment.

FIGURE 2

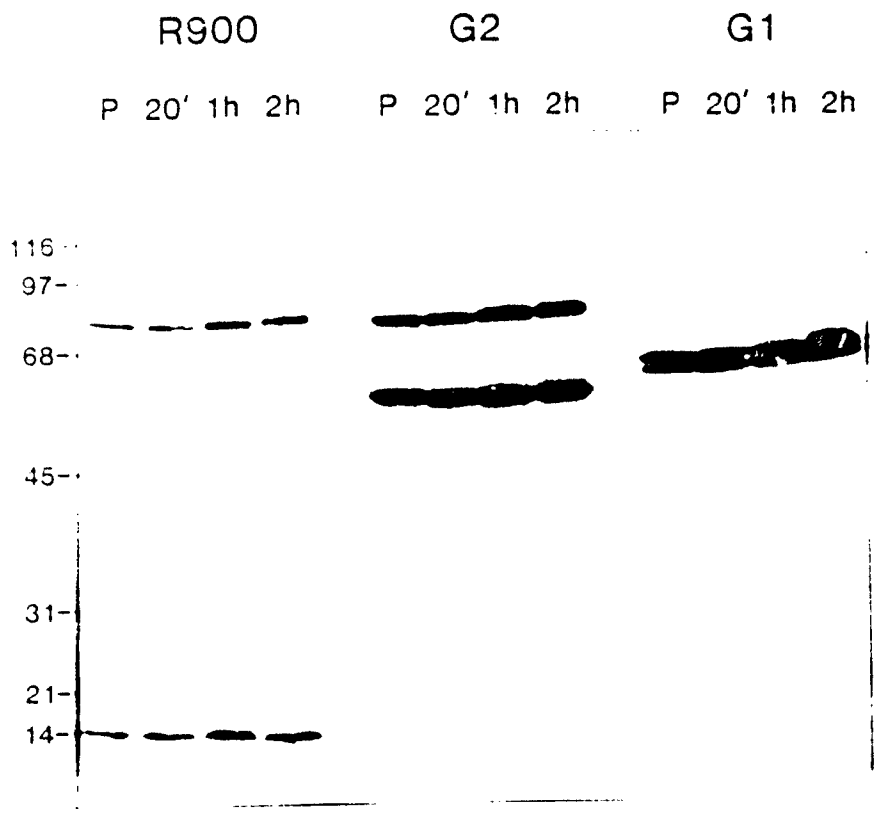

Figure 2. Pulse-chase analysis of RVFV M segment protein expression. Four dishes of BSC-40 cells were infected with a recombinant vaccinia virus 7. Three hours post-infection, cells were starved for methionine and then pulse radiolabeled with ^{35}S-methionine for 30 minutes, at which time the cells from one dish (P) were immediately harvested, while the remaining dishes were washed three times with complete culture medium lacking radiolabel but supplemented with 100-times the normal level of unlabeled methionine. Cells were incubated in this medium further supplemented with cycloheximide (30ug/ml) the indicated chase times before being harvested. Cleared lysates were prepared, and equal aliquots from each timepoint were immunoprecipitated with rabbit antisera directed against sequences within the coding region of either glycoprotein G2 (G2), glycoprotein G1 (G1), or the 12 amino acid sequence between the second and third in-phase ATG codons (R900) (2). Equal portions of the immunoprecipitated polypeptides were electrophoresed in an SDS-containing 10-15% gradient polyacrylamide gel, which was subsequently fluorographed and exposed to X-ray film. The numbers at the left indicate approximate molecular masses (in kilodaltons).

FIGURE 3

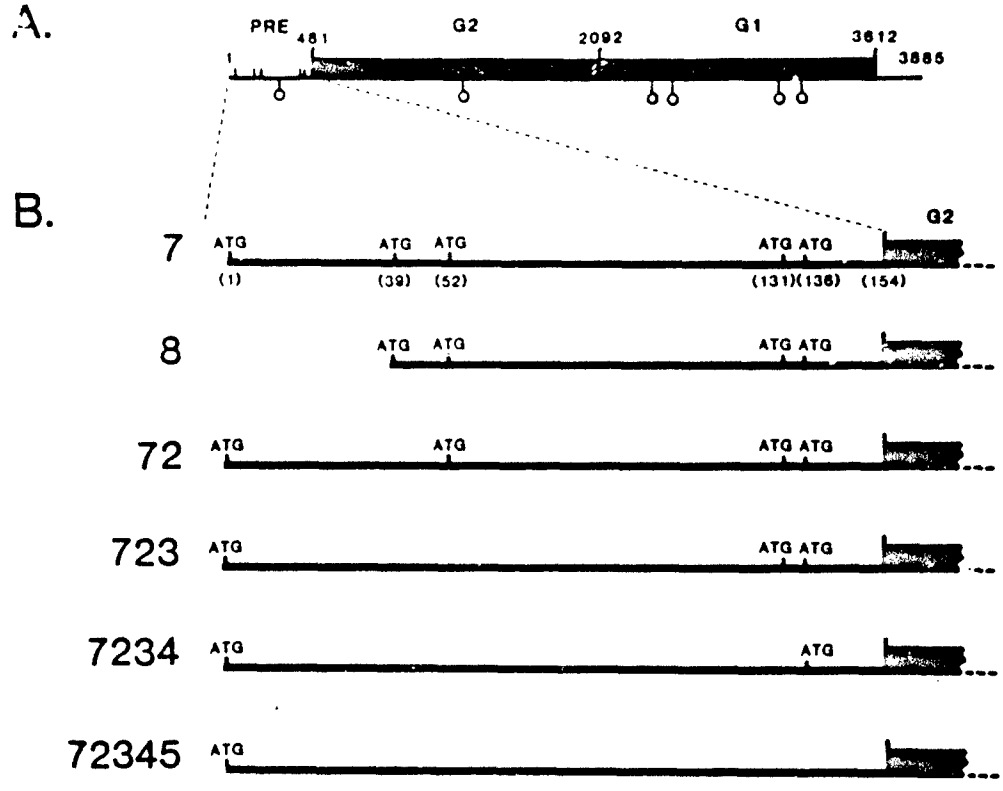

Figure 3. RVFV M segment sequences found in recombinant vaccinia viruses. Panel A. Schematic representation of the M segment as described in the legend to Fig. 1. Panel B. An expanded view of the 5' end of the M segment ORF indicating the presence and positions of the five in-phase ATG codons in the preglycoprotein region represented in various recombinant vaccinia viruses (denoted by the numbers at the left). Numbers in parentheses indicate their amino acid position within the ORF.

FIGURE 4

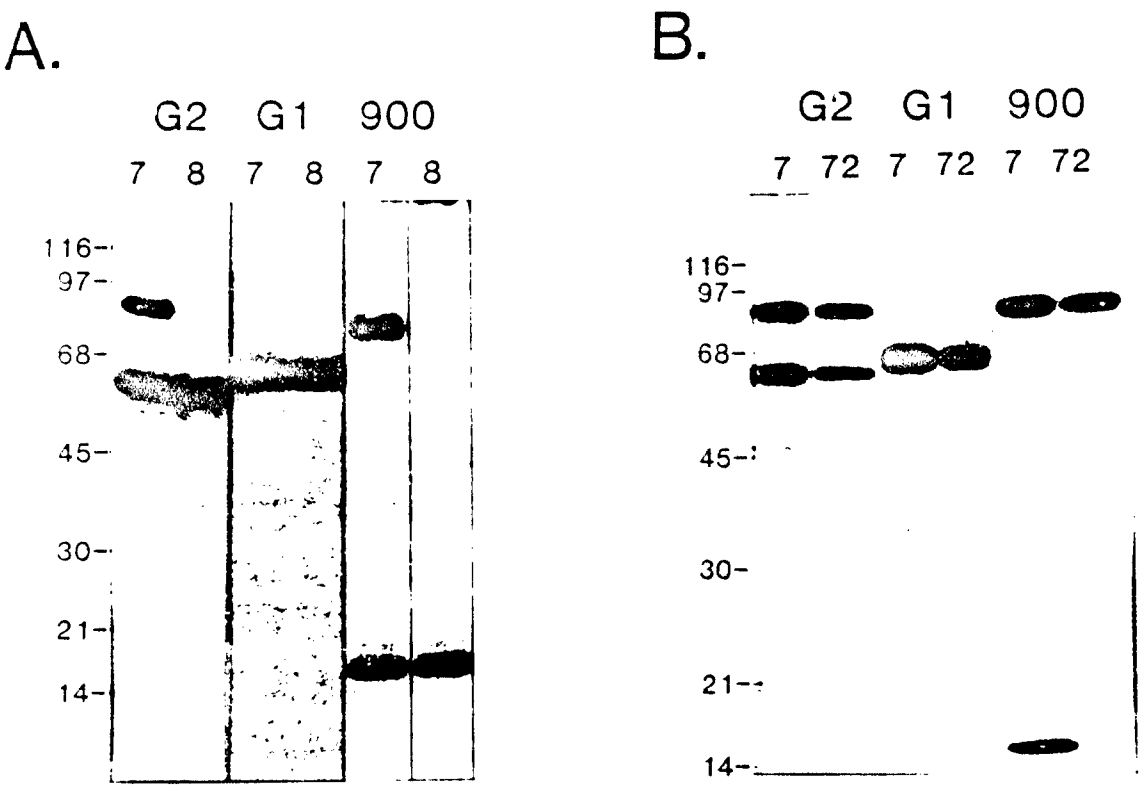

Figure 4. Role of the first and second ATG codons of the ORF on RVFV M segment protein expression. Cleared cell lysates from ^{35}S-methionine-labeled virus-infected cells were prepared for virus 7, virus 8, and virus 72. Portions of these lysates were then used for immunoprecipitation with three rabbit sera: Antiserum specific for glycoprotein G2 (G2), glycoprotein G1 (G1), and the 12 amino acid sequence between the second and third in-phase ATG codons (R900). Aliquots of the immunoprecipitated polypeptides were electrophoresed in SDS-containing 10-15% gradient polyacrylamide gels which were subsequently fluorographed and exposed to X-ray film. Panel A shows the comparison of the M segment polypeptides expressed by virus 7 and virus 8. Panel B shows a similar comparison between viruses 7 and 72. Numbers in the left margins indicated the positions of molecular mass markers (in kilodaltons).

FIGURE 5

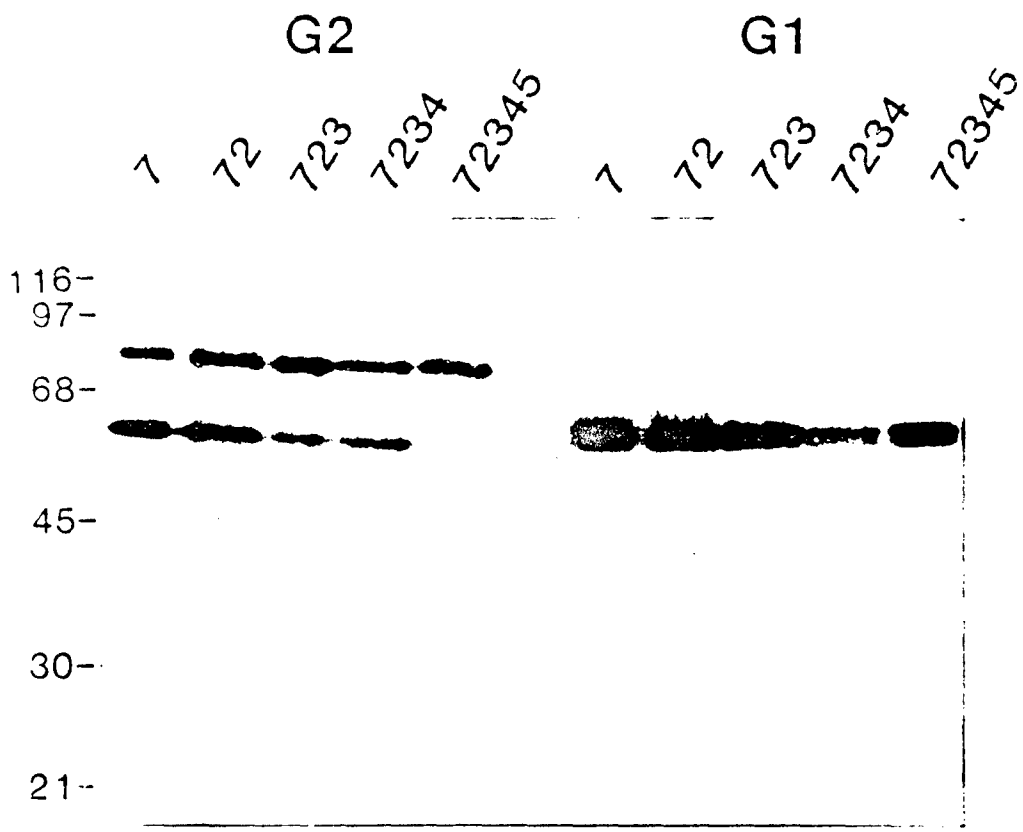

Figure 5. Effect of elimination of ATG codons within the preglycoprotein region on the expression of M segment proteins. Cleared lysates were prepared from ^{35}S-methionine-labeled cells infected with recombinant vaccinia viruses 7, 72, 723, 7234, and 72345. Proteins were immunoprecipitated with antiserum specific for glycoprotein G2 or G1. The precipitated proteins were resolved on an SDS-containing 10% polyacrylamide gel. Molecular mass standards are indicated on the left.

FIGURE 6

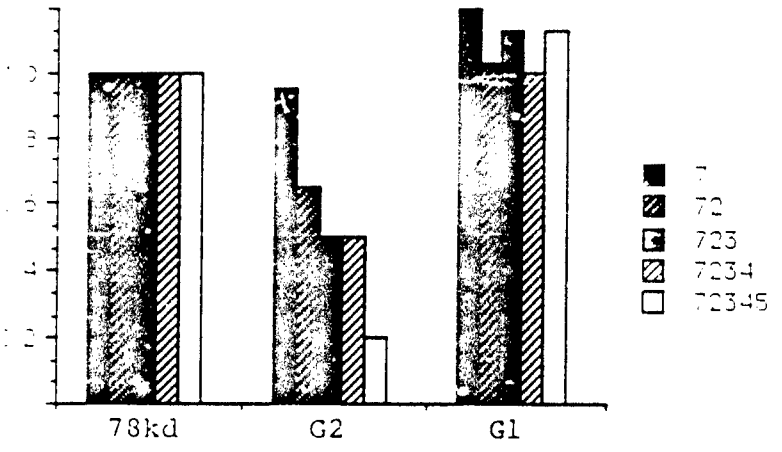

Figure 6. Relative amounts of M segment glycoprotein synthesized in cells infected with recombinant vaccinia viruses 7, 72, 723, 7234, and 72345. Regions corresponding to labeled protein bands in the gel depicted in Fig. 5 were excised and the amount of radioactivity in them was determined by scintillation counting. Counts per band were adjusted for the number of methionine residues contained in each particular protein and compared to the counts per methionine value obtained for the 78kd protein of that viral lysate. The 78kd protein numbers for each virus were normalized to a value of 1.

FIGURE 7

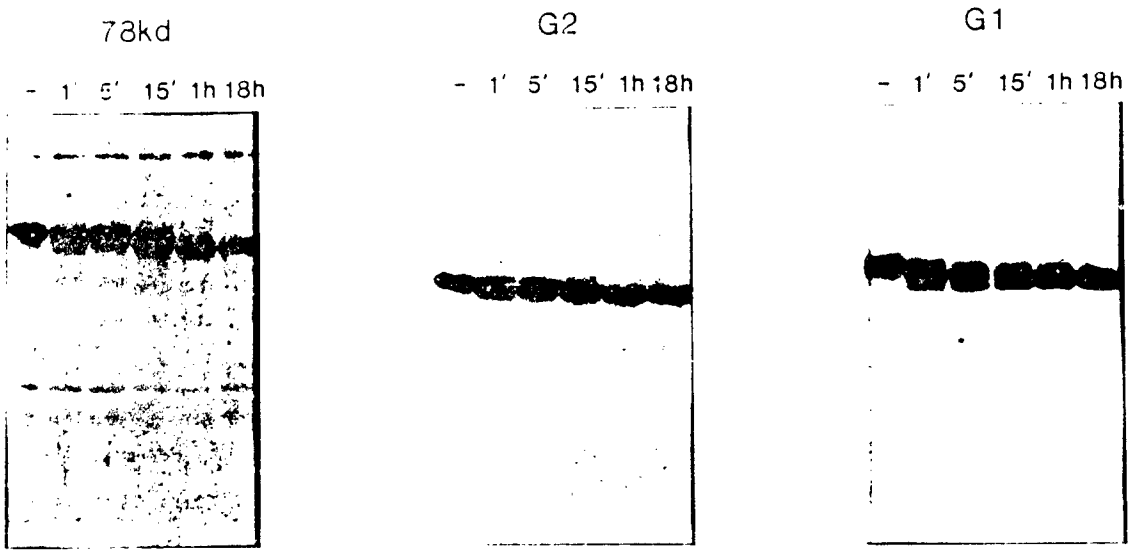

Figure 7. Time course of endoglycosidase F treatment of RVFV M segment glycoproteins. BSC40 cells were infected with either recombinant virus 7 or 6, radiolabeled with ^{35}S-methionine, and solubilized in SDS lysis buffer. The cleared cell lysates were immunoprecipitated with antiserum specific for the 78kDa protein (R970) (virus 7-infected cells), or antiserum specific for glycoprotein G2 or G1 (virus 6-infected cells). The washed immune complexes were eluted from Pansorbin and treated with endoglycosidase F for the indicated times as described in the Materials and Methods. Samples were electrophoresed in SDS-containing 7.5% (for 78kDa protein samples) or 10% (for glycoproteins G2 and G1) polyacrylamide gels adjacent to similar portions of untreated (-) material. Gels were then fluorographed and exposed to X-ray film.

FIGURE 8

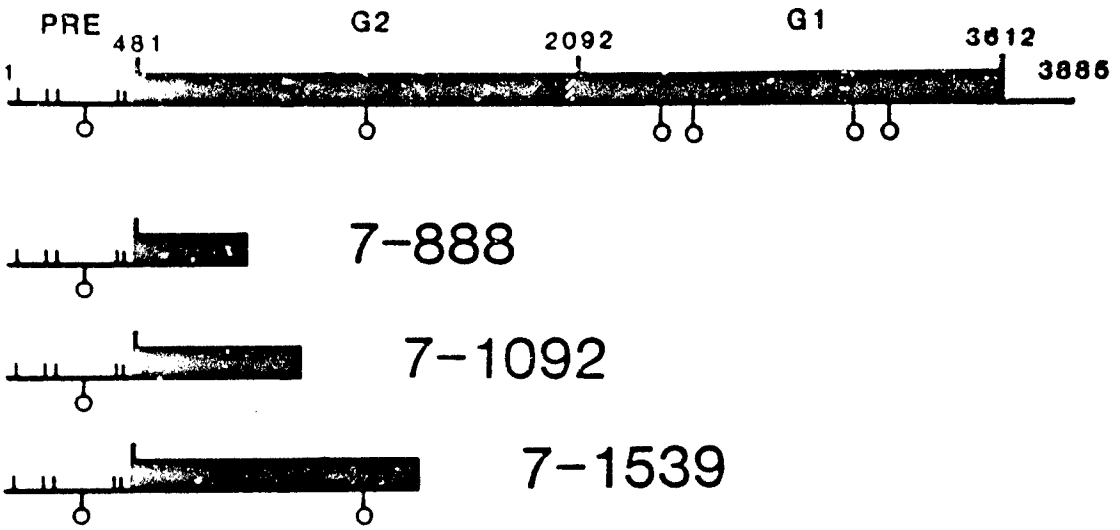

Figure 8. Schematic representation of the RVFV M segment sequences present in recombinant vaccinia viruses 7-888, 7-1092, 7-1539. The numbers and symbols are as described in the legend to Fig. 1.

FIGURE 9

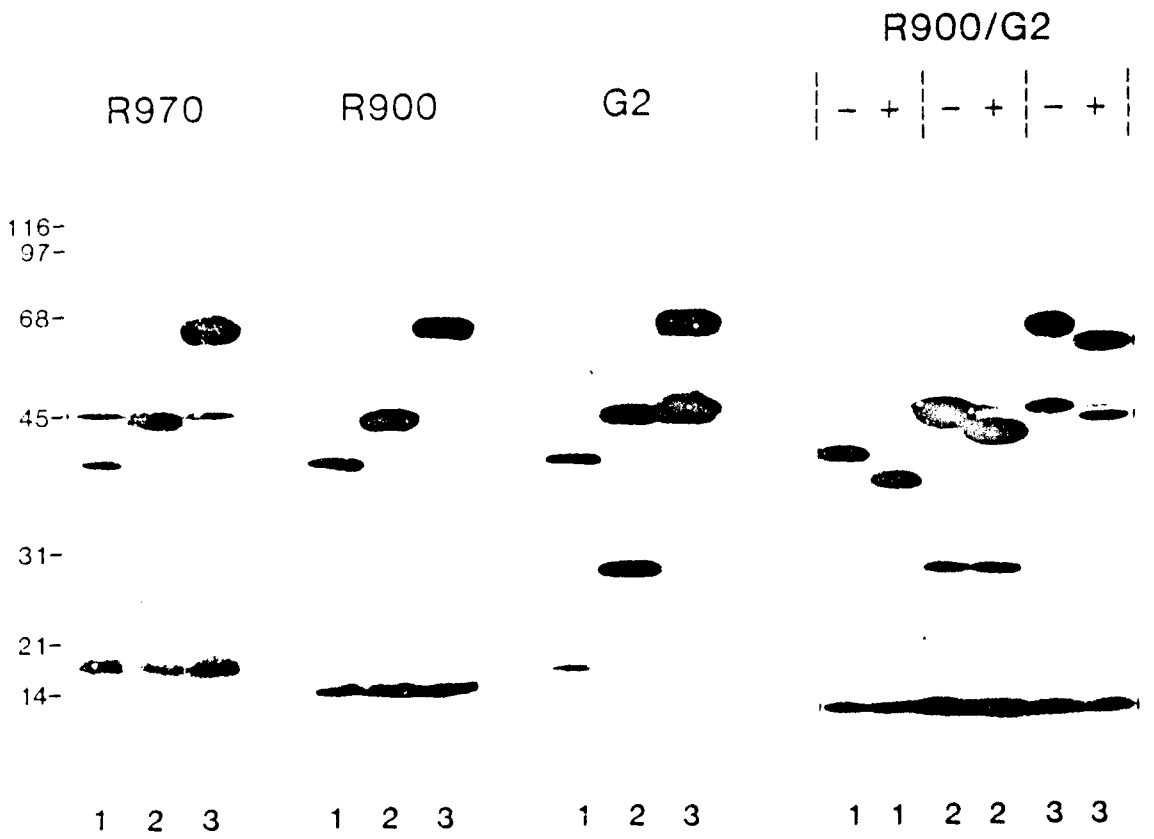

Figure 9. Polypeptides expressed in cells infected with the series of recombinant vaccinia viruses possessing truncated M segment ORFs, and their glycosylation status. Cleared SDS lysates were prepared from ^{35}S-methionine-labeled virus 7-888, 7-1092, and 7-1539 infected cells. Portions of each were immunoprecipitated with either antiserum R970 (recognizing sequences between the first and second ATG codons; first panel), R900 (recognizing sequences between the second and third ATG codons; second panel), antiserum specific for glycoprotein G2 (third panel), or a mixture of R900 and G2 antisera (R900/G2; fourth panel). Materials immunoprecipitated with R900/G2 antisera were divided: one portion was treated (+) with endoglycosidase F to effect complete deglycosylation, the other left untreated (-). Aliquots of all samples were electrophoresed in SDS-containing 10-15% polyacrylamide gradient gels. The numbers at the left indicate the molecular masses (in kilodaltons) of standards. Tracks 1: samples from virus 7-888-infected cells; tracks 2: samples from virus 7-1092-infected cells; tracks 3: samples from virus 7-1539-infected cells.

FIGURE 10

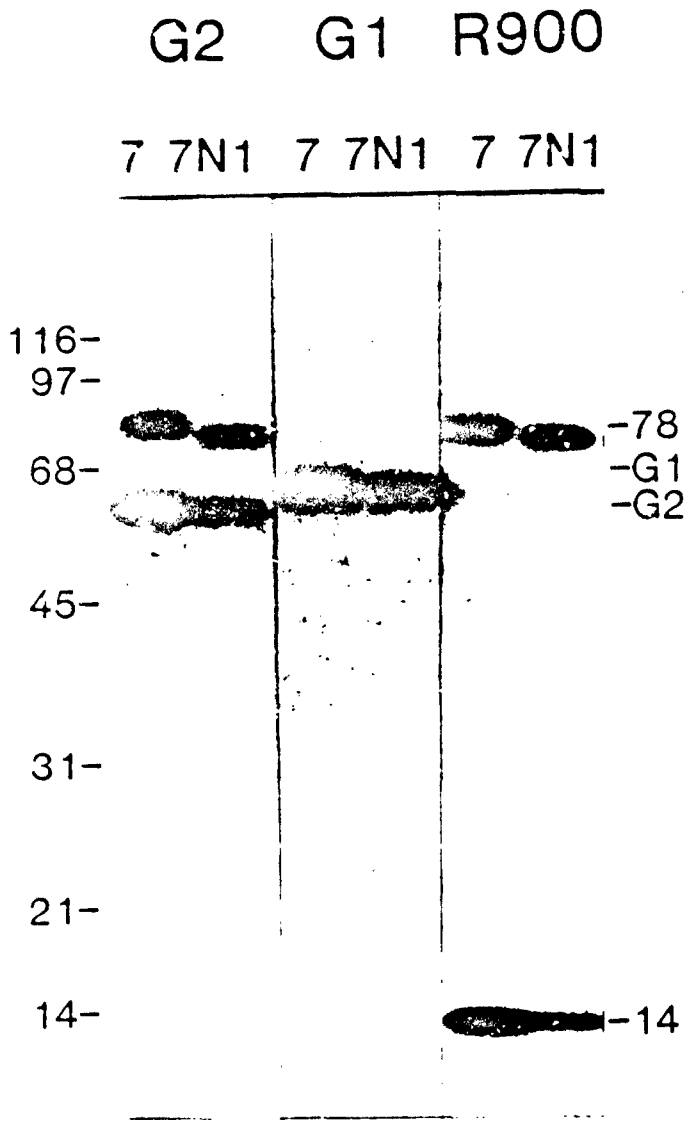

Figure 10. RVFV M segment protein expression in cells infected with recombinant virus 7N1. BSC-40 cells were infected with either recombinant vaccinia virus 7 or 7N1 and labeled with ^{35}S-methionine. Cleared SDS lysates were prepared, and aliquots were immunoprecipitated with antiserum specific for glycoprotein G2 or G1, or R900 antiserum. Immunoprecipitated proteins were analyzed on an SDS-containing 10-15% polyacrylamide gradient gel. The positions of the RVFV M segment proteins are indicated on the right. The numbers at the left show the positions of molecular mass standards.

FIGURE 11

Immunizing virus	Mouse Protection (# alive/challenged) ICR	C57B16
none	0/5	0/5
7-888	0/5	0/6
7-1092	0/6	0/6
7-1539	1/6	0/6
7-1860	5/5	6/6
7-2097	6/6	5/5

Figure 11. Mouse protection following recombinant vaccinia virus immunization.

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