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# Use of Bacterial Expression Cloning To Define the Amino Acid Sequences of Antigenic Determinants on the G2 Glycoprotein of Rift Valley Fever Virus

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**Four distinct antigenic determinants along the G2 glycoprotein encoded by the M segment RNA of the *Phlebovirus* Rift Valley fever virus were localized. These epitopes were defined by four monoclonal antibodies, three of which were capable of neutralizing virus infectivity; one was nonneutralizing. Immunoprecipitation by these monoclonal antibodies of either denatured or native antigen characterized the epitopes as having linear or higher order structure. Molecular cloning of G2 glycoprotein-coding sequences into a bacterial expression plasmid utilizing a β-galactosidase fusion protein system was employed for epitope localization. A nuclease BAL 31 plasmid expression library, in which processive regions of the 3′ end of the G2 glycoprotein coding sequences were deleted, allowed for approximation of the carboxy-terminal limit of the antigenic determinants. Further subcloning of limited G2 polypeptide sequences into the bacterial expression vector permitted more refined localization of the epitopes. The characteristics of the immunoreactivity of these small peptide regions (between 11 and 34 amino acids) produced in bacteria as G2-β-galactosidase fusion proteins were similar to those of the authentic Rift Valley fever virus G2 glycoprotein. These defined antigenic determinants and their importance in virus infectivity are discussed.**

Rift Valley fever is an important disease of domestic livestock populations in much of sub-Saharan Africa, causing acute febrile disease, abortions, and death in cattle and sheep. Rift Valley fever is also a human disease, usually causing a denguelike illness. However, the explosive epidemic of Rift Valley fever that occurred in Egypt in 1977 and 1978 was characterized by severe clinical disease in humans, including hemorrhagic syndrome, encephalitis, blindness, and death (14, 17). This Egyptian epizootic demonstrated the potential for this disease to escape its normal endemic area, and furthermore, established Rift Valley fever as a threat to human as well as livestock populations worldwide (10, 18). The etiologic agent of Rift Valley fever is Rift Valley fever virus (RVFV), a member of the *Phlebovirus* genus of the *Bunyaviridae* family (1). Members of the *Phlebovirus* genus exhibit similar structural features characteristic of all members of the *Bunyaviridae*. The genome of RVFV consists of three RNA segments designated L ($2.3 \times 10^6$ to $2.7 \times 10^6$ daltons), M ($1.38 \times 10^6$ daltons), and S ($0.5 \times 10^6$ to $0.6 \times 10^6$ daltons) (5, 20, 21). The S segment RNA of RVFV and Punta Toro virus (another member of the genus *Phlebovirus*) encodes both the viral nucleocapsid protein (N; 27,000 to 28,000 daltons) and a nonstructural polypeptide $NS_S$ (29,000 to 31,000 daltons) (11, 20). The complete nucleotide sequences of the M segment RNA of RVFV and Punta Toro have revealed that this RNA encodes the two viral glycoproteins (G1 and G2) and possibly one or more additional, yet unidentified, nonstructural polypeptides (5, 12). Although no direct evidence is available, it is presumed that the L segment RNA codes for the L protein, a component of the viral transcriptase.

An important goal of our studies on RVFV centers around devising a strategy and means for safe and effective disease prevention and protection measures. To attain this goal, it is essential to gain an understanding of basic molecular features of the virus replication strategy and virus structural protein immunology. With respect to the latter point, the glycoproteins of the Bunyavirus La Crosse and snowshoe hare (7–9) and the Phlebovirus Punta Toro (6) have been shown to induce and interact with virus-neutralizing antibodies. Polyclonal, monospecific antisera to either the G2 or G1 glycoprotein of RVFV are able to neutralize RVFV infectivity in a standard in vitro plaque-reduction neutralization test (J. Smith, J. Meegan, and J. Dalrymple, unpublished data). Furthermore, monoclonal antibodies (mAbs) have been generated to both RVFV glycoproteins that are able to similarly neutralize virus infectivity in vitro (Smith et al., unpublished data). We undertook studies to further characterize immunoreactive (both antigenic and immunogenic) sequences on the RVFV glycoproteins. Here are presented the results of a study in which the amino acid sequences of four antigenic determinants on the G2 glycoprotein, as defined by three virus-neutralizing mAbs and one nonneutralizing mAb, are identified.

## MATERIALS AND METHODS

**Antibodies.** Series of mouse mAbs to RVFV glycoproteins were generated and initially characterized by J. Meegan, J. Smith, and J. Dalrymple of the Virology Division, U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), Frederick, Md. These workers supplied us with mAbs directed against the G2 glycoprotein with the following designations: 4-32-8D, 4-D4, 3C-10, and 4-39-CC. In this report they will be referred to as mAbs I, II, III, and IV, respectively. mAbs I, II, and IV were able to neutralize virus infectivity, as determined in vitro in a plaque-reduction neutralization test. Furthermore, these mAbs, when passively administered to mice, were able to protect animals against lethal virus challenge (Smith et al., unpublished data). mAb III was neither virus neutralizing in vitro nor protective

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in mice. mAbs unreactive with the RVFV G2 glycoprotein (used as controls) included a mAb to a bacterial pilus protein (provided by P. Sadowski of Molecular Genetics, Inc.) or a mAb to the G1 glycoprotein of RVFV (provided by J. Smith of USAMRIID). Hyperimmune guinea pig serum to RVFV was provided by J. Dalrymple of USAMRIID.

**Radiolabeling of cultures, immunoreactivity analyses, and protein analyses.** Experiments involving the use of infectious RVFV were carried out in the P3+ containment facilities of the Virology Division of USAMRIID. The Zagazig human strain (ZH501) of RVFV (17) was used to infect Vero cell monolayers. At 12 h postinfection, cultures 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. Radiolabeled cells were harvested and disrupted by one of two procedures. For standard lysis, cells were suspended at 4°C in the detergent-containing RIPA buffer (2) supplemented with 0.5 M KCl. An alternate procedure was done by suspending cells in sodium dodecyl sulfate buffer (10 mM Tris hydrochloride [pH 7.2], 1 mM EDTA, 1% SDS, 10 mM dithiothreitol) and boiling for 5 min, followed by a 10-fold dilution with buffer H (10 mM Tris hydrochloride [pH 7.2], 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, 0.05% deoxycholate). All lysates were cleared at 16,000 × *g* for 20 min.

To radiolabel bacterial cell proteins, cultures grown in methionine-free minimal medium were incubated for 30 min with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.) and then supplemented with 100 μCi of [$^{35}$S]methionine per ml and harvested after 2 min. Standard cell lysis involved treatment of bacterial cells with lysozyme (5 mg/ml for 10 min at 4°), followed by dilution into RIPA buffer, sonication, incubation for 10 min at 4°C with Pansorbin (Calbiochem-Behring, La Jolla, Calif.) and by clearing at 16,000 × *g* for 10 min. Alternatively, pelleted bacterial cells were suspended in SDS buffer, boiled for 5 min, diluted with buffer H, and then incubated with Pansorbin and cleared. The lysates that were cleared as described above were immunoprecipitated with the various antibodies by previously described procedures (2). Samples of radiolabeled, immunoprecipitated polypeptides were electrophoresed in SDS-containing 7.5 or 10% polyacrylamide gels (13), fluorographed (4), and exposed to x-ray film.

Total bacterial lysates were generated by suspending pelleted cells in SDS gel sample buffer (13) and boiling for 5 min. After gel electrophoresis, the resolved polypeptides were either stained with Coomassie blue or electrophoretically transferred from the gel to nitrocellulose filters, incubated with appropriate mAbs, and probed with $^{125}$I-labeled protein A (Bolton-Hunter reagent; Amersham, Corp., Arlington Heights, Ill.) as described previously (3).

**Cloning techniques.** Restriction enzymes (New England Biolabs, Inc., Beverly, Mass.) were used as specified by the manufacturer. DNA fragments were resolved by electrophoresis in 1% low-melting-point agarose gels (15), localized by ethidium bromide fluorescence, and excised. Select DNA fragments in molten low-melting-point agarose gel slices were ligated into the appropriately restricted β-galactosidase expression vector pTC413 with T4 DNA ligase (New England Biolabs) at room temperature for 16 h. pTC413, a derivative of pJS413 (23, 24), contained the *tac* promoter (22), the ribosome binding site and the first 23 codons of the phage λ *cro* gene followed by a *Bgl*II, *Hin*dIII, *Sma*I, and *Bam*HI polylinker which allowed for insertion of sequences in phase with a β-galactosidase *i/z* gene fusion (see Fig. 2D). For all ligations, the appropriately restricted vector was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described by Maniatis et al. (15). Ligation mixtures were transformed into *Escherichia coli* NF1829 (23, 24) by standard procedures (15) and plated on MacConkey agar indicator plates (19) containing 100 μg of ampicillin per ml. Colonies that were red, that is, that exhibited β-glactosidase activity, were analyzed.

**Generation of nuclease BAL 31 expression libraries.** BAL 31 nuclease (New England Biolabs) digestion was carried out at 37°C in a solution composed of 0.6 M NaCl–15 mM $CaCl_2$–15 mM $MgCl_2$–20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA at an enzyme concentration of 0.5 U/μg of linearized plasmid DNA. Fractions were removed every 45 s over 7 min and extracted with phenol saturated with 150 mM NaCl–10 mM Tris hydrochloride (pH 7.2)–1 mM EDTA followed by chloroform extraction and ethanol precipitation. The ends of the resultant fragments were repaired with DNA polymerase I (Klenow fragment; New England Biolabs) as described previously (15). A second restriction enzyme digestion was performed to release from the plasmid backbone the nuclease-treated fragments, which were subsequently resolved by electrophoresis in a low-melting-point agarose gel. The resulting distribution of fragments was excised, ligated to *Sma*I-restricted pTC413, transformed into *E. coli* NF1829, and plated on MacConkey agar plates as described above. Sizes of plasmid DNA inserts were determined after appropriate restriction enzyme digestion by electrophoresis in 8% polyacrylamide gels (15). DNA sequencing was performed by the method of Maxam and Gilbert (16).

## RESULTS

**Immunoreactivity of mAbs to the G2 glycoprotein of RVFV.** A standard immunoprecipitation of [$^{35}$S]methionine-labeled RVFV-infected Vero cell lysates with antisera from a RVFV-hyperimmunized guinea pig reveals reactivity with three major polypeptides: the two glycoproteins of RVFV, G1 and G2 (glycoprotein G1 often appears as a doublet [5]), and the viral nucleocapsid protein N (Fig. 1A). A similar immunoprecipitation employing four mAbs to glycoprotein G2 (I, II, III, and IV) demonstrated their reactivity and specificity for this protein (Fig. 1B). The immunoprecipitations described above were performed with cell lysates generated by a standard detergent lysis method. In an alternate approach, cells were disrupted by boiling in SDS and dithiothreitol prior to dilution and immunoprecipitation. Presumably this procedure results in the denaturation of antigens. When such a lysate was prepared and immunoprecipitated with the four mAbs, only mAbs I, II, and III were still able to recognize the G2 glycoprotein; mAb IV was unable to react with the denatured protein (Fig. 1C).

**Expression of G2 glycoprotein sequences in *E. coli*.** To characterize the glycoprotein sequences involved in antibody recognition by these four mAbs, portions of the G2 glycoprotein gene were engineered so that they could be expressed as fusion polypeptides in *E. coli*. In Fig. 2A is shown the genetic organization and mature glycoprotein coding regions of the M segment RNA of RVFV. An expanded view of the G2 glycoprotein gene and adjacent regions, with relevant restriction endonuclease sites, is presented in Fig. 2B. Two bacterial expression plasmids were constructed initially. Plasmid ptRV-BHF was made by ligating a *Bam*HI-*Hin*dIII restriction fragment (nucleotides 302 to 1092; Fig. 2B) excised from a cDNA clone of the M segment (5) to the expression vector pTC413 (Fig. 2D)

restricted with *Bgl*II and *Hin*dIII. When introduced into *E. coli*, the resulting plasmid (ptRV-BHF) directed by the synthesis of a *cro*-G2-β-galactosidase fusion protein with a molecular weight of 147,000 which was designated BH. The BH protein encoded the amino-terminal 43% of the mature G2 glycoprotein sequence, as well as 59 amino acids of the RVFV polypeptide preceding these mature sequences (Fig. 2C).

The RVFV sequences inserted into a second expression construct were generated by BAL-31 nuclease digestion of a cDNA clone linearized with *Hpa*I at nucleotide coordinate 2145, followed by restriction with *Bam*HI at nucleotide 302 (Fig. 2B). The population of DNA fragments generated in this way was ligated to pTC413 restricted with *Bgl*II and *Sma*I. After transformation of *E. coli* and screening, a colony containing a plasmid designated ptRV-C12F was found that expressed a 172,000-molecular weight *cro*-G2-β-galactosidase fusion protein termed C12. The G2 glycoprotein sequences represented in this construct included nucleotides 302 through 1770, encompassing the amino-terminal 91% of the mature G2 protein (Fig. 2C).

**Antigenicity of the bacterially expressed G2 glycoprotein analogs.** To establish that the bacterially expressed G2 glycoprotein analog C12 possessed the antigenic determinants defined by the four mAbs under study, the following experiment was performed. *E. coli* harboring the plasmid ptRV-C12F were induced to express the C12 protein and were then radiolabeled with [$^{35}$S]methionine. A standard detergent lysate was generated and used for immunoprecipitation. All four mAbs specifically immunoprecipitated the 172,000-molecular-weight C12 polypeptide (Fig. 3A). When radiolabeled bacterial cell lysates were prepared by boiling in SDS and dithiothreitol and then immunoprecipitated, the C12 protein was still recognized by mAbs I, II, and III, but not by mAb IV (Fig. 3B). Thus, the bacterial C12 fusion polypeptide possessed and presented the protein sequences necessary for recognition by the four mAbs in a manner similar to that in the authentic viral G2 glycoprotein (Fig. 1A and B). The numerous smaller polypeptide bands observed in specific immunoprecipitates most likely represent immunoreactive degradation products of the fusion protein.

To approximate the location of the antigenic determinants defined by these mAbs, [$^{35}$S]methionine-labeled bacterial

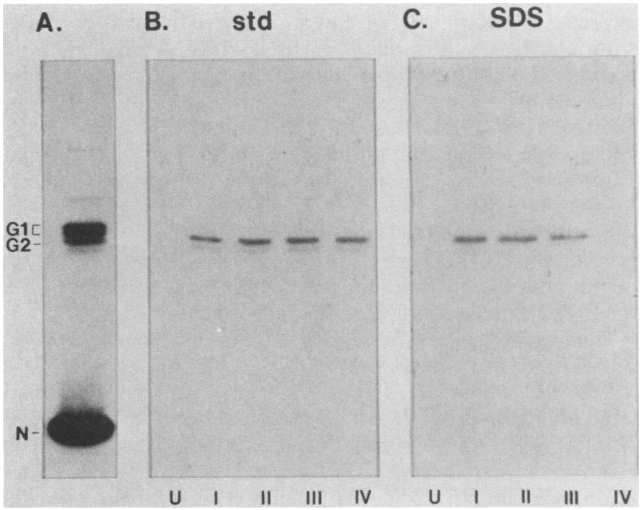


FIG. 1. Immunoreactivity of mAbs to the G2 glycoprotein of RVFV. (A) [$^{35}$S]methionine-labeled RVFV-infected Vero cell lysates were generated by standard detergent lysis and immunoprecipitated with antiserum from a RVFV-hyperimmunized guinea pig. Three major proteins were recognized: G1 glycoprotein (appearing as a doublet), G2 glycoprotein, and the viral nucleocapsid protein N. (B) An identical radiolabeled standard detergent lysate (std) was immunoprecipitated with an unrelated mAb (U) and four RVFV mAbs (I, II, III, and IV). (C) A [$^{35}$S]methionine-labeled RVFV-infected Vero cell lysate generated by an SDS lysis method (see text) was immunoprecipitated with an unrelated mAb (U) and mAbs I, II, III, and IV.

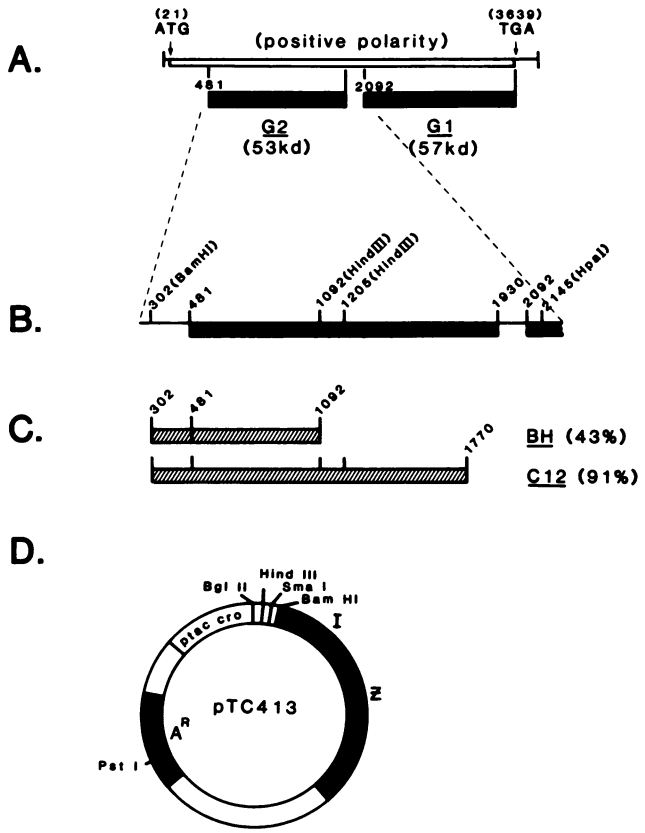


FIG. 2. Expression of the G2 glycoprotein in *E. coli*. (A) Molecular organization of the RVFV M segment RNA. The major open reading frame in the viral complementary polarity is presented. Nucleotide coordinates for the first initiation codon, the stop codon of the major open reading frame, and G1 and G2 glycoproteins are given. The solid bars represent the coding sequences of the mature glycoproteins. Their nonglycosylated molecular sizes (in kilodaltons [kd]) are given. (B) G2 glycoprotein coding region and surrounding sequences showing nucleotide coordinates of restriction enzyme sites relevant to the construction of RVFV bacterial expression plasmids. (C) Portions of G2 glycoprotein expressed in *E. coli*. The shaded bars represent portions of mature G2 glycoprotein-coding regions and surrounding sequences which were cloned into a plasmid expression vector (panel D). The resultant cro-G2-β-galactosidase fusion proteins, designated BH and C12, expressed 43 and 91%, respectively, of the mature G2 glycoprotein coding sequences. (D) The bacterial expression vector pTC413. All bacterial expression plasmids described in this report were constructed with pTC413. This vector, derived from pJS413 (23, 24), contained the β-lactamase gene ($A^r$), the *tac* promoter-operator (22), and 23 amino acids of the phage λ *cro* gene. These sequences were followed by a polylinker region containing the unique restriction enzyme sites *Bgl*II, *Hin*dIII, *Sma*I, and *Bam*HI and an *i/z* gene fusion encoding β-galactosidase. The diagrams in panels A through D are not drawn to scale.

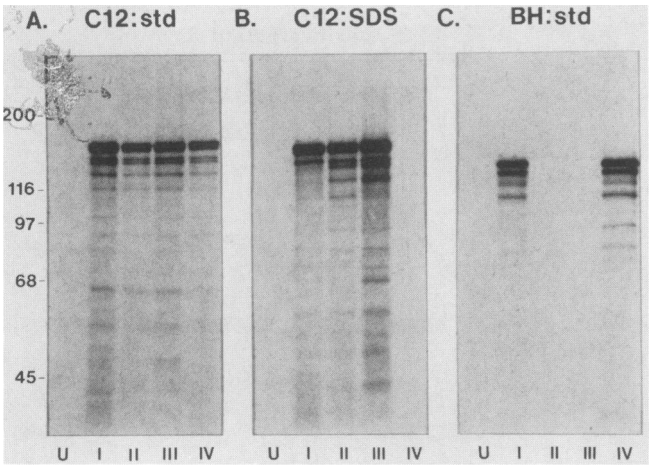


FIG. 3. Antigenicity of bacterially expressed G2 glycoprotein analogs. Bacteria harboring a plasmid (ptRV-C12F) directing the synthesis of the C12 fusion protein were labeled with [$^{35}$S] methionine and used to generate a standard (std) detergent lysate (A) and an SDS lysate (B). (C) Bacteria harboring a plasmid (ptRV-BHF) directing the synthesis of the BH fusion protein were similarly radiolabeled and used to generate a standard detergent lysate (std). Portions of these lysates were immunoprecipitated with an unrelated mAb (U) and four mAbs against the G2 glycoprotein (I, II, III, and IV). The numbers in the left margin represent the positions of molecular size standards (in kilodaltons).

cells containing the plasmid ptRV-BHF and expressing the BH fusion protein were used to generate standard detergent lysates for immunoprecipitation. Figure 3C shows that only mAbs I and IV were able to immunoprecipitate the BH protein, indicating that the antigenic determinants defined by these two mAbs resided in the amino-terminal 43% of the G2 glycoprotein. The lack of reactivity to the BH protein by mAbs II and III (Fig. 3C), coupled with their recognition of the C12 protein, positioned their epitopic regions between the amino-terminal 43 and 91% of the mature G2 glycoprotein.

**Establishing the carboxy-terminal limits of the antigenic determinants defined by the mAbs.** The data presented above only allowed for a rough localization of the antigenic determinants of the four mAbs. To refine these positions, expression plasmids were generated in which processive portions of the carboxy terminus of the G2 protein-coding sequences were deleted. This was carried out with BAL-31 nuclease. BAL 31 nuclease deletions were generated from either the unique *Hin*dIII site (nucleotide 1092) of plasmid ptRV-BHF (for mapping epitopes defined by mAbs I and IV) or the unique vector site *Bam*HI outside the G2 glycoprotein carboxy-terminal sequences in plasmid ptRV-C12F (for mapping of the epitopes defined by mAbs II and III). In both cases carboxy-terminal-deleted fragments were excised from the plasmids by *Pst*I restriction (Fig. 2D). The resultant population of DNA fragments (from the *Pst*I site in the β-lactamase gene to the variable BAL-31 nuclease termini in the G2 glycoprotein gene) was ligated to pTC413 restricted with *Pst*I and *Sma*I (Fig. 2D). Ligation mixtures were transformed into *E. coli*, and colonies expressing β-galactosidase fusion proteins were selected. Plasmids from individual colonies were screened for RVFV insert size by restriction enzyme analysis (data not shown). Figure 4A displays total bacterial cell protein profiles from ordered sets of induced cultures of these BAL 31 nuclease expression clones, as resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. The clones are numbered such that clones with higher numbers contain larger portions of the G2 protein sequence (or are deleted less). Expression clones 1 through 9 were generated by BAL 31 nuclease treatment at the *Hin*dIII site in ptRV-BHF, and clones 10 through 21 were generated by nuclease digestion from the *Bam*HI site in ptRV-C12F.

Once these sets of BAL 31 nuclease carboxy-terminal deletion constructs were established, the reactivity of the mAbs with the expressed polypeptides could be evaluated. Immunoreactivity of mAbs I, II, and III with the respective sets of expression clone proteins was carried out in a Western immunoblot format (Fig. 4B). However, because reactivity with mAb IV appeared to be sensitive to antigen denaturation (Fig. 1C and 3B), radiolabeled detergent lysates of the appropriate deletion clones were generated and used in a standard immunoprecipitation analysis (Fig. 4B). The last reactive (lowest numbered) protein in each set of expression clones defined a maximum limit for the carboxy-terminal end of each antigenic determinant. That is to say that clone 4 expressed the smallest portion of the G2 protein sequences that was still recognized by mAb I. Similarly, clone 12 expressed the smallest polypeptide reactive with mAb II, clone 18 expressed the smallest protein reactive with mAb III, and clone 6 expressed the smallest protein recognized by mAb IV (Fig. 4B).

**Further definition of the antigenic determinants recognized by the mAbs.** The plasmids of the last immunoreactive clones shown in Fig. 4 were DNA sequenced to establish the precise nucleotide-amino acid position within the G2 glycoprotein gene represented in each construct. With knowledge of the limit of the carboxy-terminal sequences sufficient for antibody recognition by each of the mAbs, we attempted to define the amino-terminal limit of the epitopes. Specific sequences from the plasmids of the last reactive clones described in Fig. 4 were excised and subcloned into the expression vector pTC413. A characteristic of the expression constructs described in this study is that they all possessed a unique *Bam*HI site in the pTC413 vector region immediately downstream from the inserted RVFV sequences (Fig. 2D). Thus, for subcloning, restriction at this vector *Bam*HI site and convenient sites upstream from that point was used to excise regions of the G2 protein sequence from the respective clones. These DNA fragments were subsequently isolated and cloned into the appropriately restricted pTC413 expression vector. The immunoreactivity of the new fusion proteins produced by bacteria harboring these constructs could then be evaluated.

Approximately 106 base pairs upstream from the vector *Bam*HI site in clone 4 was an *Alu*I site at nucleotide 790 in the RVFV sequence. This *Alu*I-*Bam*HI fragment was isolated and ligated to pTC413, which had been restricted with *Hin*dIII (Fig. 2D); and the 5′ end overhang was filled in by DNA polymerase and then cleaved with *Bam*HI. When introduced into *E. coli*, the resulting plasmid directed the synthesis of a *cro*-G2-β-galactosidase fusion protein, designated AB-4, which contained 34 amino acids from the G2 glycoprotein (amino acids 105 through 138 in the mature G2 protein sequence; Fig. 5A). This fusion polypeptide was specifically immunoprecipitated from bacterial cell lysates prepared by the SDS method with mAb I (Fig. 5B, lanes 1 and 2). In an attempt to further refine the localization of the mAb I epitope, two additional DNA fragments were subcloned in phase in pTC413: an *Alu*I-*Dde*I fragment which encompassed amino acids 105 through 127, and a *Dde*I-*Bam*HI fragment which included amino acids 121 through

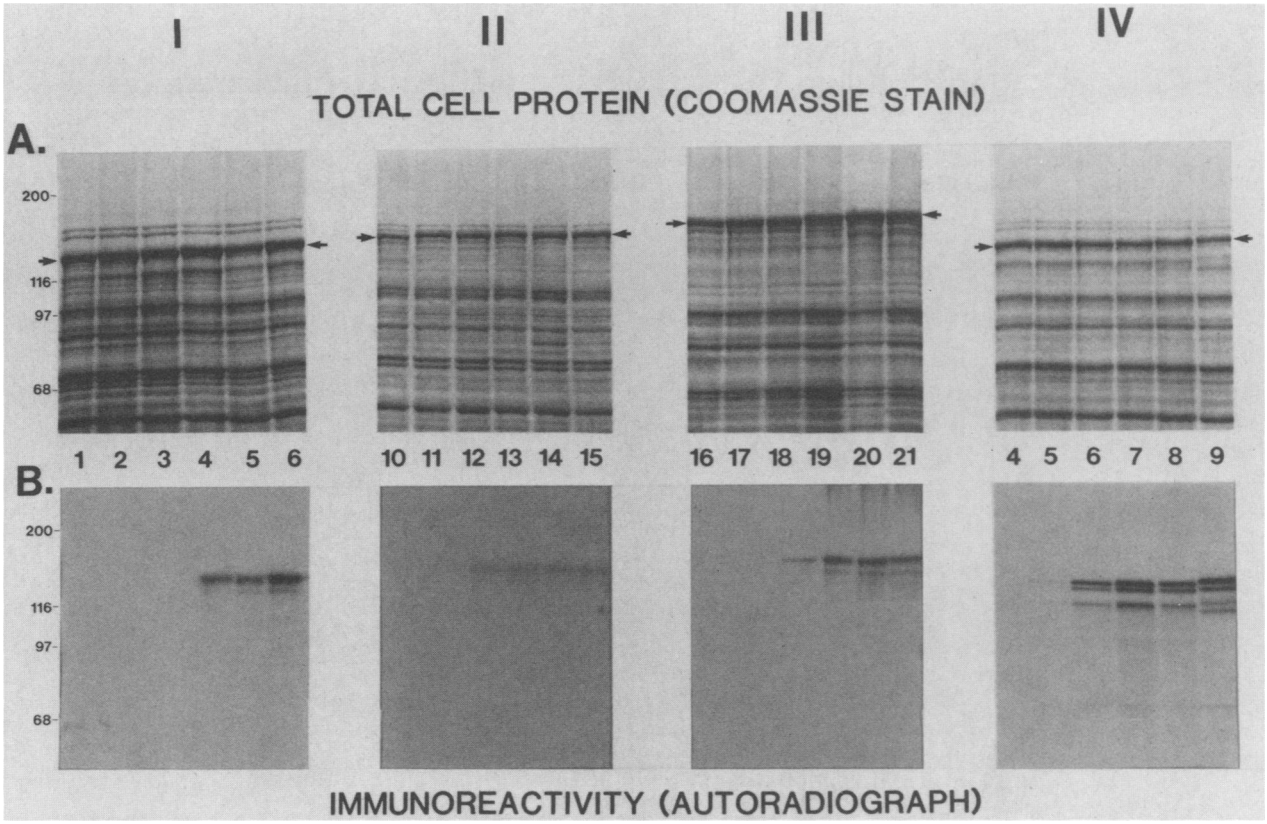


FIG. 4. Definition of the carboxy-terminal limits of the antigenic determinants recognized by the mAbs. (A) Total cell protein of bacteria harboring nuclease BAL 31 expression plasmids. Bacteria containing expression plasmids that were deleted for varying portions of the carboxy terminus of the glycoprotein G2 were induced to synthesize *cro*-G2-β-galactosidase fusion proteins. The bacteria were lysed by boiling in SDS gel sample buffer. The proteins were then resolved by SDS-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie blue. Expression clones were numbered (between the panels) such that the clones with smaller numbers had more of the carboxy terminus of G2 proteins sequences deleted than the expression clones with larger numbers. Arrows denote the position of the expressed *cro*-G2-β-galactosidase fusion proteins. (B) Immunoreactivity of the fusion proteins from the nuclease BAL 31 expression clones. The immunoreactivity of mAbs I, II, and III with total cell protein from ordered sets of expression clones was determined by Western immunoblot as described in the text. Reactivity with mAb IV was carried out by immunoprecipitation of radiolabeled standard detergent lysates generated from individual expression clones. The numbers in the left margin represent the positions of molecular size standards (in kilodaltons).

138 (Fig. 5A). Neither of the fusion proteins produced by clones carrying these constructs was immunoprecipitable with mAb I (data not shown). Therefore, although clone AB-4 defines a maximum of 34 amino acids comprising the determinant recognized by mAb I, the actual epitope is much smaller and most likely spans the two *Dde*I sites, as shown by the open bar in Fig. 5A.

To precisely define the determinant recognized by mAb II, a DNA fragment was generated from clone 12 by restriction at a *Rsa*I site at nucleotide 1162 in the RVFV sequence and the *Bam*HI vector site. The 39-base-pair fragment was cloned into pTC413 (*Hin*dIII restricted, DNA polymerase filled in, *Bam*HI restricted); and the resulting construct, when introduced into *E. coli*, directed the synthesis of a *cro*-G2-β-galactosidase fusion protein termed RsB. This polypeptide possessed 11 amino acids (from amino acid position 229 to 239) of the G2 protein (Fig. 5A) and was specifically recognized by mAb II (Fig. 5B, lanes 7 and 8).

The antigenic determinant recognized by mAb III was further localized in the following manner. First, a DNA fragment from the vector *Bam*HI site to an *Eco*RV site at nucleotide position 1607 in the RVFV sequence was excised from the last reactive clone 18 (Fig. 5A) and ligated to pTC413 cleaved at *Sma*I and *Bam*HI (Fig. 2D). The resultant construct directed the synthesis of a *cro*-G2-β-galactosidase fusion protein expressing amino acids 375 to 386 in the G2 glycoprotein sequence. However, mAb III was unable to immunoprecipitate this protein (data not shown). So we went on to clone a *Sau*3A-*Eco*RV fragment, encompassing the RVFV nucleotide sequence from 1561 to 1606, into pTC413 (Fig. 5A). The resulting plasmid directed the synthesis of a *cro*-G2-β-galactosidase protein termed SRV, expressing G2 protein amino acids 362 through 375 (Fig. 5A). This protein was specifically immunoprecipitated by mAb III (Fig. 5B, lanes 9 and 10).

Finally, to localize the epitope defined by mAb IV, a *Dde*I-*Bam*HI fragment from the last reactive clone 6 (Fig. 5A) was inserted into the vector pTC413. This plasmid construct directed the synthesis of a fusion protein designated DB-6 and expressed G2 protein amino acids 127 through 146. When radiolabeled detergent lysates from bacteria expressing DB-6 were used for immunoprecipitation, the fusion protein was specifically recognized by mAb IV (Fig. 5B, lanes 3 and 4). However, when these same bacteria expressing the DB-6 protein were prepared for immunoprecipitation by the SDS lysis procedure, mAb IV was unable to recognize the polypeptide (Fig. 5B, lanes 5 and 6). Thus, the 20-amino-acid sequence of the G2 protein represented in the

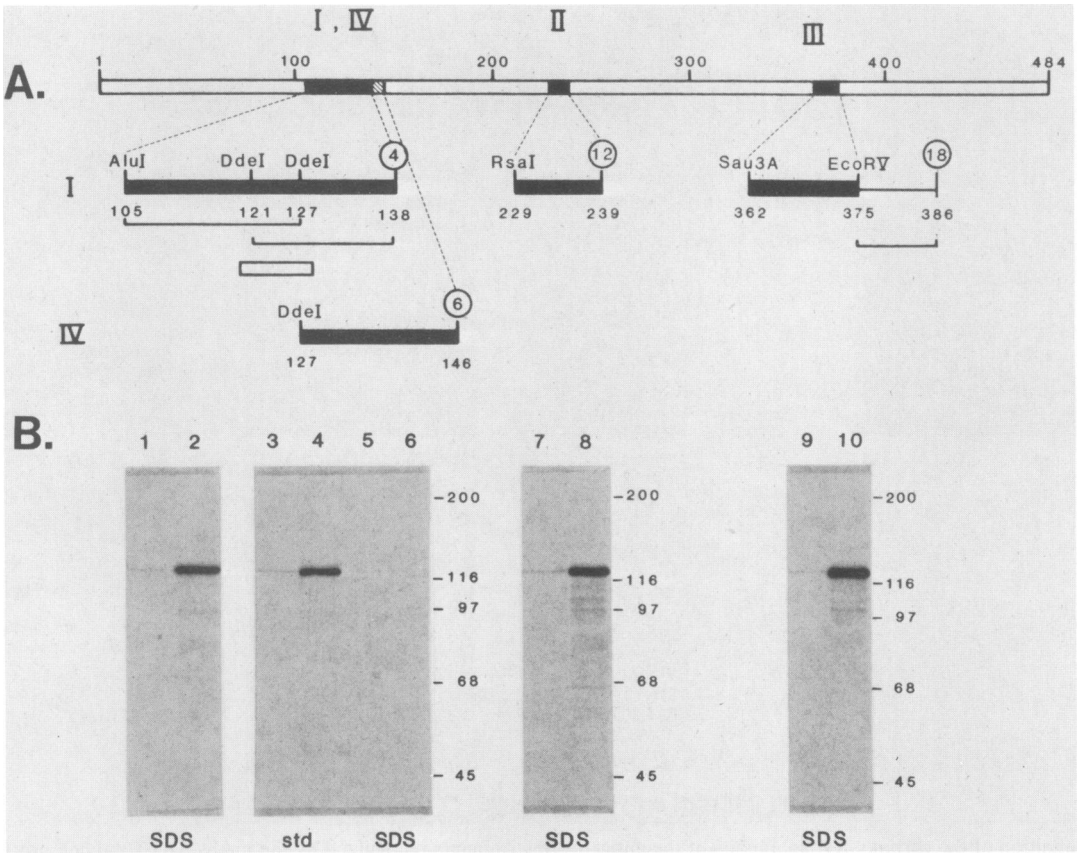


FIG. 5. Antigenic determinants along the G2 glycoprotein of RVFV. (A) Schematic diagram of the location of epitopes along the G2 glycoprotein. The alignment of the antigenic determinants defined by mAbs I, II, III, and IV with the mature G2 polypeptide is presented. The circled numbers represent nuclease BAL 31 expression clone numbers (Fig. 4); all other numbers are amino acid coordinates of the mature G2 glycoprotein. Positions of restriction sites relevant to the subcloning of RVFV sequences are shown. The solid bars represent the subcloned sequences which were immunoreactive with the indicated mAb. The thin lines represent subcloned G2 polypeptide sequences that were not immunoreactive with the mAb. The open bar signifies a proposed region of immunoreactivity. (B) Immunoprecipitation of [$^{35}$S]methionine-labeled bacterial cell lysates with mAbs. Bacteria harboring various plasmids, induced to synthesize *cro*-G2-β-galactosidase fusion proteins, were radiolabeled, and lysates were generated either by the standard detergent lysis method (std) or the SDS method. Lanes 1 and 2, immunoprecipitation of bacteria expressing AB4 with an unrelated mAb and mAb I, respectively; lanes 3 through 6, immunoprecipitation of bacteria producing DB-6 with an unrelated mAb (lanes 3 and 6) and mAb IV (lanes 4 and 5); lanes 7 and 8, immunoprecipitation of bacteria expressing RsB with an unrelated mAb and mAb II, respectively; lanes 9 and 10, immunoprecipitation of bacteria expressing SRV with an unrelated mAb and mAb III, respectively. The numbers in the right margins represent molecular size standards (in kilodaltons).

DB-6 fusion protein expressed in *E. coli* retained the structural features required for mAb IV recognition.

## DISCUSSION

The G2 glycoprotein of RVFV is undoubtedly important for viral infection and pathogenesis because this polypeptide is by itself able to induce virus-neutralizing and protective antibodies when used as an immunogen. Furthermore, mAbs to the G2 glycoprotein can be virus neutralizing and can also passively protect animals against lethal RVFV challenge (Smith et al., unpublished data). A detailed description of the immunoreactive sequences in the G2 protein, particularly those involved in recognition by virus-neutralizing antibodies, would be of extreme value in designing potential subunit vaccines to protect against RVFV infection.

We had available a battery of mAbs to the RVFV G2 glycoprotein. We selected four mAbs for the epitope mapping studies reported here: mAbs I, II, and IV were virus-neutralizing mAbs, and mAb III was nonneutralizing. Our strategy to localize the sequences involved in antibody recognition by these mAbs was the following. First, we characterized the reactivity of the mAbs with the G2 polypeptide from virus-infected cells by immunoprecipitation of the antigen prepared in two ways. A detergent lysis method presented the G2 protein in a native state, while an SDS lysis procedure generated a denatured antigen, allowing us to distinguish between antibody recognition of linear and higher order structural determinants in a superficial way. Next, we molecularly cloned a major portion (91%) of the G2 glycoprotein gene into a plasmid expression vector that allowed for the synthesis of a G2 polypeptide-β-galactosidase fusion protein in *E. coli*. We then established that each of the mAbs reacted with this bacterially produced fusion protein in the same fashion as with the authentic antigen. To actually localize the antibody recognition sequences for each mAb, nuclease BAL 31 was employed to generate plasmid expression libraries in which the 3′ end of the G2 protein gene sequences was processively removed, resulting in the production of a series of β-galactosidase fusion proteins lacking to varying extents the carboxy ter-

minus of the G2 polypeptide. Such expression libraries, when screened for immunoreactivity, allowed us to define the carboxy-end limit of the respective epitopes. Finally, both to locate the amino-terminal limit of each epitope and to establish the sequences required for antibody recognition, convenient regions including, and upstream from, the defined COOH terminus of the determinants were isolated, subcloned, and expressed as β-galactosidase fusion proteins. The smallest subcloned sequences that were reactive with the mAbs defined the maximum bounds of the epitopes.

There are several advantages to the epitope mapping approach described here. By cloning and expressing the gene sequences of interest in bacteria, we obviate the need to use authentic viral antigen. This may be an important consideration if the protein of interest is available in only limited quantities in virus-infected cells, or as in the case of RVFV, the virus itself is a pathogen requiring special handling and containment procedures. An *E. coli* plasmid expression system utilizing β-galactosidase fusion proteins greatly simplifies the selection of bacterial colonies expressing the sequences of interest. Perhaps the greatest amount of work required in this approach concerned the generation of an ordered set of BAL 31 nuclease expression clones. Once expression clones were identified, they had to be ordered. This involved plasmid isolation, careful restriction fragment sizing, and, in some cases, DNA sequencing to unequivocally establish the processive order of the deleted sequences. However, once such a BAL 31 nuclease deletion library was established, determinants defined by any number of mAbs could easily and rapidly be mapped. Furthermore, this could be done with very little antibody. Localization of the epitope defined by mAb I required less than 20 μl of ascitic fluid. Finally, no expensive equipment was required for any aspect of this approach.

However, there are also disadvantages to the approach. The entire DNA sequence of the gene of interest is necessary. Much of the final definition of the epitope relies on the convenient location of restriction sites for subcloning purposes. As seen in our mapping of the epitope defined by mAb I, such sites were not always positioned conveniently with respect to the determinants. Furthermore, this procedure defines only the maximum limits of an epitope. The actual sequences necessary for antibody recognition may be a subset within these limits.

A summary of the location within the mature G2 glycoprotein of the four antigenic determinants is presented in Fig. 5A. The nonneutralizing mAb III defines a linear epitope located toward the carboxy end of the molecule. We have found that an additional independent nonneutralizing mAb (designated 1-F11-3) also maps to this same location (data not shown). The significance of this region of the G2 protein is not known. The remaining three mAbs (I, II, and IV), however, define regions of known importance on the G2 polypeptide, because these mAbs are all able to neutralize virus infectivity. Epitopes defined by mAbs I and II appeared to be linear determinants, while epitope IV (defined by mAb IV) seemed to require some higher order structure, because antibody recognition was sensitive to antigen denaturation. Epitopes I and IV either overlapped one another or were very closely juxtaposed (Fig. 5A). Of interest in this regard is the fact that we mapped two additional, independently generated, virus-neutralizing mAbs to this same domain in the G2 polypeptide (data not shown). One mAb, designated 1-20-9C, recognized the linear sequences defined by mAb I. Another mAb, termed 4-10-10A, reacted in the same denaturation-sensitive manner as mAb IV with the DB-6 protein. mAb II defined a second functionally important region near the center of the G2 polypeptide (Fig. 5A). The fact that we mapped epitopes defined by four independent virus-neutralizing mAbs to one domain in the G2 protein, and a fifth mAb to a second region, does not exclude the possibility that additional regions within the G2 glycoprotein exist that are important for virus infectivity. Mapping of the determinants defined by additional mAbs should identify these if they are present.

TABLE 1. Amino acid sequence in the region of the mapped epitopes[a]

| Epitope | Sequence |
|---|---|
| | 105 121 127 138 |
| I | ...HYLNNDGKMASVKCPPKYGLTEDCNFCRQMTGAS |
| | 229 239 |
| II | ...KGTMDSGQTKR |
| | 362 375 |
| III | ...SQSPSTEITLKYPG |
| | 127 146 |
| IV | ...DCNFCRQMTGASLKKGSYPL |

[a] Epitope as defined by reactivity with mAbs I, II, III, and IV. Numbers represent amino acid positions in the mature G2 glycoprotein.

Table 1 provides the amino acid sequence of each determinant denoted by solid bars in Fig. 5A. Because these sequences define only the maximum limits of the respective antibody recognition sites, we are cautious to draw any conclusions concerning the local structure of each determinant. A hydropathic plot of the entire G2 polypeptide sequence has been presented elsewhere (5). The determinant regions defined by mAbs I and IV and by mAb II fall in areas of the protein that are predicted to have an overall hydrophilic nature (5). This is reflected in the amino acid composition of the sequences shown in Table 1: epitope I, 8 charged, 16 polar, and 10 nonpolar amino acids; epitope II, 4 charged, 6 polar, and 1 nonpolar amino acid; epitope IV, 4 charged, 10 polar, and 6 nonpolar amino acids. The region of the polypeptide in which epitope III is located consists of 2 charged, 8 polar, and 4 nonpolar amino acids and is predicted to have a neutral hydropathic nature (5).

The epitope defined by mAb IV is of interest in that antibody recognition appeared to require some structural feature of the 20 contiguous amino acids. It was somewhat surprising that such structure was maintained in the context of the large β-galactosidase fusion polypeptide. One possibility for this maintenance of structure considers the potential stabilizing effects of a putative disulfide bridge between cysteines 128 and 131 in the RVFV sequence (Table 1). Also interesting is the potential contribution of proline 145 (Table 1). The first nonreactive fusion polypeptide, expressed in clone 5 (Fig. 4), was identical in sequence to DB-6, with the exception that it lacked proline 145 and leucine 146 of the RVFV sequence. The elimination of these two amino acids reduced the immunoreactivity of the polypeptide by 80%.

Studies to address features required for the structural integrity of epitope IV are under way. In addition, further definition of the necessary amino acid sequences for antibody recognition is proceeding with the use of synthetic peptides. Results of this study and the mapping of antigenic determinants defined by additional mAbs will aid in describing the functional role of specific amino acid sequences and

regions of the G2 glycoprotein of RVFV that are important in virus infectivity and pathogenesis.

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