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# Localization of the Virus Neutralizing and Hemagglutinin Epitopes of E1 Glycoprotein of Rubella Virus

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Current serological assays using whole rubella virus (RV) as a target antigen for detecting RV-specific antibodies fail to define specific RV proteins and antigenic determinants such as hemagglutinin (HA) and virus-neutralizing (VN) epitopes of rubella virus. A panel of E1 deletion mutants and a subset of E1-specific monoclonal antibodies (MAb) were used for the initial analysis of HA and VN epitopes of E1 glycoprotein. A peptide region ($E1_{193}$ to $E1_{269}$) was found to contain HA and VN epitopes. Using both overlapping synthetic peptides and truncated fusion proteins within this region, the HA epitope defined by MAb 3D9F mapped to amino acid residues $E1_{214}$ to $E1_{240}$, while two VN epitopes defined by MAb 21B9H and MAb 16A10E mapped to amino acid residues $E1_{214}$ to $E1_{233}$ and $E1_{219}$ to $E1_{233}$, respectively. The epitopes defined in this study are recognized by antibody whether or not the epitopes are glycosylated. 

## INTRODUCTION

Rubella virus (RV), the causative agent of German measles, is a small enveloped RNA virus in the Togavirus family (Porterfield *et al.*, 1978). Its genome consists of a single-stranded 40 S RNA of positive polarity (Oker-Blom *et al.*, 1984). In addition to the 40 S genomic RNA, RV-infected cells contain a subgenomic 24 S RNA derived from the 3′ end of the 40 S RNA (Oker-Blom *et al.*, 1984). The translation of the 24 S subgenomic RNA produces a 110-kDa precusor polyprotein that is proteolytically processed to yield three structural proteins, C, E2, and E1 (Oker-Blom *et al.*, 1983). The capsid protein C is a nonglycosylated protein of 33 kDa rich in basic amino acids and proline (Clarke *et al.*, 1987). E1 (58 kDa) and E2 (42–47 kDa) are both type 1 membrane glycoproteins found on the virion surface as viral spikes (Oker-Blom *et al.*, 1983). Studies with monoclonal antibodies (MAbs) suggest that E1 is the major target for antibodies (Waxham and Wolinsky, 1987). E2 is topologically buried under E1 on the viral surface (Ho-Terry and Cohen, 1984) and plays a role in the cell surface expression of E1 (Hobman *et al.*, 1990).

Although clinical rubella is a relatively mild disease, RV remains an important human pathogen because of its teratogenic effects (Oxford and Obery, 1985). Complications such as polyarticular arthralgia and arthritis following vaccination or infection are common (Chantler *et al.*, 1982) and rare cases of progressive panecephalitis have also been reported (Marvin, 1975). At present serological techniques with whole RV as a target antigen for the detection of antibodies to RV are most commonly used for laboratory diagnosis of acute and congenital rubella infections and for determination of rubella immunity. These assays lack defined specificity against antigenic determinants such as hemagglutinin and virus-neutralizing epitopes of RV. For example, women, seronegative as measured by hemagglutination inhibition assay (<1:8), were shown to have moderate levels of RV-specific antibodies, measurable by enzyme-linked immunosorbent assays (ELISAs) using whole RV (Tingle *et al.*, 1983). Sera from congenital rubella syndrome patients have high levels of antibodies directed to E2, but with low or no reactivity to E1 (Chaye *et al.*, 1992). ELISAs employing whole RV fail to distinguish between the various antibody specificities. Therefore, it is necessary to define the functional epitopes of RV structural proteins for diagnostic assays in order to assess immunity against RV infection as well as to aid the development of noninfectious rubella vaccines.

Six independent epitopes have been identified within RV E1 protein. These epitopes include domains that are important for viral infectivity and hemagglutination (Green and Dorsett, 1986, Ho-Terry *et al.*, 1985, Waxham and Wolinsky, 1987). Epitopes that react with MAbs specific for hemagglutination (HA) and virus neutralization (VN) have been localized to 41 amino acid residues ($E1_{245}$ to $E1_{285}$) (Terry *et al.*, 1988), and to 82 amino acid residues ($E1_{202}$ to $E1_{283}$) (Wolinsky *et al.*, 1991) of RV E1 protein, respectively.

In this study, we describe the closer localization of

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HA and VN epitopes of RV E1 glycoprotein. A panel of E1 deletion mutants and a subset of E1-specific MAbs were used for analysis of HA and VN epitopes of E1 protein. A peptide domain of 77 amino acid residues ($E1_{193}$ to $E1_{269}$) was found to contain HA and VN epitopes. Overlapping peptides within this region were used to further refine the HA and VN epitopes. The HA epitope was mapped to 27 amino acid residues ($E1_{214}$ to $E1_{240}$) and two VN epitopes were localized to amino acid residues $E1_{214}$ to $E1_{233}$ and $E1_{219}$ to $E1_{233}$, respectively.

## MATERIALS AND METHODS

### General recombinant DNA techniques

Restriction endonucleases and other DNA-modifying enzymes were purchased from commercial sources and used according to manufacturers' specifications. Standard methods were used for the construction, amplification, and purification of the plasmids (Maniatis *et al.,* 1982).

### Construction of E1 deletion and truncation mutants

A series of in-frame deleted and truncated cDNAs (Fig. 1) was generated by using restriction sites within the E1 coding sequence in p3′ E2/E1 plasmid (Hobman *et al.,* 1988). The constructed mutants were confirmed by restriction analysis and/or by DNA sequencing (Sanger *et al.,* 1977).

Deletion mutants were generated as follows:

(1) m1: the *Xho*I fragment (450 nt) was excised from plasmid p3′ E2/E1 and the product religated.

(2) m2: the fragment (560 nt) from *Bam*HI to *Hin*dIII sites was removed from p3′ E2/E1 and the product religated.

(3) m3: the fragment (670 nt) from *Sma*I to *Hin*dIII sites was removed, the ends filled by repair, and religated.

(4) m4: the fragment (1057 nt) from *Nco*I to *Sma*I sites was removed, the ends filled by repair, and religated.

(5) m5: the fragment (1147 nt) from *Nco*I to *Bam*HI sites was removed, the ends filled by repair, and religated in the presence of *Bam*HI linker (pGGGATCCC) to introduce the correct reading frame.

(6) m6: the fragment (670 nt) from *Sma*I to *Hin*dIII sites was excised from m1, the ends filled by repair, and religated.

(7) m7: the fragment (787 nt) from *Nco*I to *Xho*I sites was excised from m6, the ends filled by repair, and religated.

(8) FP-1 to FP-5: cDNA fragments were amplified by the polymerase chain reaction (Erlich, 1989) using synthetic oligonucleotides (as shown below), subcloned into PET3xb vector (Studier *et al.,* 1990), and sequenced to check for mutations which may have accumulated during the amplification. The synthetic oligonucleotides used in the amplification were:

FP1 (CCATGGGGAACCAACAGTCCCGGT and CCATGGGGGACGCTCTGGCGT),
FP2 (CCATGGGGGAGGTCCAGGTCCCG and CCATGGATGACAATTCGGGCTCC),
FP3 (CCATGGGGCATGGCCCCGATTGGGC and CCATGGGGGACGCTCTGGCGT),
FP4 (CCATGGGGAACCAACAAGTCCCGGT and GCCAACGCCACTCCCCTGACT),
FP5 (CCATGGGGAACCAACAGTCCCGGT and CCATGGATGACAATTCGGGCTCC).

### Polymerase chain reaction (PCR)

E1 fragments of FP1 to FP5 were amplified by PCR using a DNA thermal cycler (Perkin–Elmer Cetus). PCR mixtures contained 50 m*M* KCl, 10 m*M* Tris–HCl (pH 8.3), 15 m*M* $MgCl_2$, 200 $\mu M$ each of the four dNTPs, 0.01% gelatin, 0.1% Triton-X 100, 2 units of Taq DNA polymerase (Bio-Can), 1 $\mu$l of p3′ E2/E1 plasmid, and 1 $\mu$l of 10 m*M* primers. Thermal cycle parameters were 95° for 2 min, 60° for 30 sec, 72° for 1 min, for a total of 30 cycles. PCR amplified products were gel purified, treated with T4 DNA polymerase, and ligated to pET3xb vector (Studier *et al.,* 1990) that had been digested with *Bam*HI and the ends filled by repair.

### *In vitro* transcription and translation

For cell free expression, p3′ E2/E1 and the deleted/truncated constructs in pSPT19 were linearized with *Hin*dIII (wild-type E1, m4, and m5), *Sma*I (m2), or *Bam*HI (m3), respectively. Linearized DNA templates were transcribed with SP6 RNA polymerase (Promega) as described (Hobman *et al.,* 1988). Translation of SP6-derived transcripts was performed in a nuclease-treated rabbit reticulocyte lysate system (Promega) containing 0.02 m*M* amino acid mixture minus cysteine, [$^{35}$S]cysteine (1.2 mCi/ml), RNasin (1600 U/ml), and RNA (40 $\mu$g/ml) in a volume of 25 $\mu$l. After 1 hr incubation at 30°, translated products were immunoprecipitated with human anti-RV serum or monoclonal antibodies, separated on 10% SDS–PAGE (Laemmli, 1970), and analyzed by fluorography.

### Expression in COS cells

Mutants to be expressed in COS cells were subcloned into the vector pCMV5 (Andersson *et al.,* 1989). E1 and m5 cDNAs were subcloned between *Eco*RI and *Hin*dIII sites and m3 between *Eco*RI and *Sma*I sites.

Mutant m2 was constructed from pCMV5-E1 (Hobman *et al.*, 1990) by removing the 560-nt fragment from *Bam*HI in E1 cDNA to *Bam*HI in the multiple cloning site of the vector and religating the product. COS cells were transfected with constructed plasmid cDNAs according to methods described previously (Hobman *et al.*, 1988). After 48 hr transfection, the transfected COS cells were scraped off the plates and analyzed by immunoblotting.

### Expression in *Escherichia coli*

*E. coli* expression vectors pET8c; pET3a,b,c; and pET3xa,b,c were provided by Dr. F. W. Studier (Brookhaven National Laboratory, Upton, New York). All pET translation vectors place cloned cDNA under control of both T7 promoter and an efficient translation initiation signal for gene 10 protein of T7 phage (Rosenberg *et al.*, 1987). Vectors pET3a,b,c carry a fragment that codes for the first 11 amino acids of the gene 10 resulting in a fusion protein (Studier *et al.*, 1990). The letters "a", "b", and "c" of the pET3 and pET3x vectors denote the three reading frames relative to the gene 10 initiation codon. Vector pET8c will not contain any of the gene 10 protein. Translation products from vectors pET3xa, xb, and xc are fusion proteins with 261 amino acid residues from the amino-terminus of the gene 10. These vectors allow expression of small foreign proteins (<10 kDa) that may be unstable in the host strain.

Expression of the truncated derivatives of E1 was directed by inducible T7 RNA polymerase engineered in the *E. coli* strain BL21(DE3)/pLysS (Rosenberg *et al.*, 1987). This strain contains a copy of T7 RNA polymerase gene located in the chromosome under the control of the inducible lacUV5 promoter. Cultures were grown at 37° in L-broth containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). T7 RNA polymerase was induced by addition of isopropylthiogalactoside (IPTG) (0.04 m*M*) when the culture reached an optical density of 0.8–0.99 at 600 nm.

Induced cultures were allowed to grow for an additional 2–4 hr at 37° and subsequently harvested by centrifugation. The pellets were resuspended in 1/50 volume of DNase I buffer (50 m*M* Tris–HCl, 5 m*M* EDTA, 10 m*M* $MgSO_4$), freeze/thawed twice, and then treated with DNase I (1 mg/ml) for 15 min at room temperature. Samples (5–10 μl) were analyzed by electrophoresis on 12% SDS–PAGE. Expressed proteins were detected by immunoblotting. Expression of recombinants from pET3x vectors was sufficiently high to be visualized by Coomasie blue staining. Bands corresponding to recombinant proteins were cut out and electroeluted for 3 hr at 10 mA (Electroeluter 422, Bio-Rad, Richmond, CA). The eluates were lyophilized and solubilized in 8 *M* urea containing 50 m*M* Tris–HCl, pH 7.5, 5 m*M* EDTA. Supernatant solutions were collected following centrifugation to remove insoluble material and analyzed on immunoblots.

TABLE 1

SUMMARY OF PROPERTIES OF MONOCLONAL ANTIBODIES DIRECTED AGAINST RV E1 GLYCOPROTEIN

| Antibody | Isotype | HAI[a] | VN[b] |
|---|---|---|---|
| 3D9F | IgG2b | 16384 | – |
| 3D5D | IgG2b | 8192 | – |
| 12B2D | IgG2a | 4096 | + |
| 21B9H | IgG1 | <8 | + |
| 16A10E | IgG1 | 32 | + |

[a] HAI, hemagglutination inhibition assay.

[b] VN, plaque reduction assay; (+) more than 50% plaque reduction at 1:100 dilution of monoclonal antibody, (–) less than 50% plaque reduction at 1:100 dilution of monoclonal antibody.

### Generation of monoclonal antibodies (MAbs)

Four-week-old Balb/C mice were immunized by intraperitoneal injection of purified RA-27/3 strain RV (500 hemagglutinin (HA) units/mouse) in Freund's complete adjuvant. Five injections of 250 HA units/mouse were administered at 3-week intervals. Finally, 500 HA units/mouse in saline were administered 3 days before fusion. Immune spleen cells were fused with NS-1 myeloma cells using polyethylene glycol 1500 (Galgre *et al.*, 1977). Supernatants were screened for the presence of rubella-specific antibodies by ELISA, and cells from positive wells were subsequently cloned twice by single-cell dilution cloning.

Ascites fluids were purified using the Affi-gel protein A MAPS II system (Bio-Rad). The isotypes of MAbs were determined by double immunodiffusion against a set of standard goat anti-mouse sera (Tago, Burlingame, CA). Hemagglutinin inhibition (HI) assays were performed using a heparin/manganous chloride procedure (Libhaber, 1970). Virus neutralization was determined by immunocytochemical focus assays using peroxidase-conjugated rabbit immunoglobulin (Ig) to mouse IgG (Fukuda *et al.*, 1987). Of the 25 MAbs 3D9F, 3D5D, and 12B2D were characterized to have HI activity of 1:16384, 1:8192, and 1:4096, respectively. 21B9H, 12B2D, and 16A10E were found to have VN activity (Table I).

### Immunoblot analysis

The RV proteins separated on polyacrylamide–SDS gels were transferred onto a nitrocellulose membrane (Hybond-C, Amersham). The membrane was washed

for 10 min in TBS (183 m*M* NaCl, 13 m*M* Tris–HCl, pH 7.5) plus 0.3% Tween-20 and then blocked for 30 min in 4% BSA in TBS, followed by 2 hr incubation at room temperature with either MAbs of appropriate dilutions or human anti-RV serum. Positive bands were visualized using alkaline phosphatase-conjugated anti-human or anti-mouse IgG (Gibco-BRL).

### Peptide synthesis

Peptides were synthesized in an automated ABI 430A peptide synthesizer using solid-phase methods (Merrifield, 1969). Eight overlapping peptides (length 15–35 residues) covering the region (residues 193 to 269) of RV E1 protein were synthesized. Synthetic peptides were cleaved from the resin by treatment with hydrogen fluoride and purified by reversed-phase high-pressure liquid chromatography using a Vydac C4 column. The purity of all peptide preparations exceeded 95%. For each peptide, amino acid analyses were performed on a Waters Pico-Tag system and found to be in good agreement with the theoretical composition.

### Peptide-specific ELISA

E1 synthetic peptides (1 $\mu$g/well) were coated onto Immulon-2 plates (Dynatech) in carbonate buffer (15 m*M* $Na_2CO_3$, 35 m*M* $NaHCO_3$, pH 9.5) overnight at room temperature. Following 1 hr blocking in PBS containing 0.5% skim milk, the plates were incubated with MAbs diluted in PBS–0.5% skim milk. After 2 hr incubation the plates were washed and alkaline phosphatase-conjugated goat anti-mouse IgG antibody (BRL) (diluted 1:3000) was added. The plates were developed in buffered substrate (1 *M* diethanolamine, 5 m*M* $MgCl_2$, *p*-nitrophenylphosphate 92 mg/ml, pH 9.6) and read at 405 nm on a microplate reader (Bio-Rad).

## RESULTS

### Mapping the peptide regions containing HA and VN epitopes

The initial localization of the peptide regions containing HA and VN activities was carried out by construction of deletion mutants (m1, m2, and m3) using restriction sites within the E1 coding sequence in p3′ E2/E1 plasmid (Hobman *et al.,* 1988). The wild-type E1 construct p3′ E2/E1, in addition to containing the entire E1 gene, also contains the capsid protein translation start site as well as nucleotides specifying the first eight amino acids of C and 69 carboxyl-terminal residues of E2, including the putative E1 signal sequence (Hobman *et al.,* 1988). The cDNA inserts from p3′ E2E1, m1, m2, and m3 were subcloned into the eukaryotic expression vector pCMV5 (Andersson *et al.,* 1989), downstream from the human cytomegalovirus immediate early gene promoter. COS cells were transfected with recombinant plasmids and cell lysates were isolated 48 hr post-transfection for immunoblot analysis. Wild-type E1 and mutants (m1, m2, and m3) all reacted with MAbs that exhibit VN (Fig. 2A) and HAI activities (Fig. 2B). The positive recognition of mutants m1, m2, and m3 by both MAbs suggests that the HA and VN epitopes are not contained within the *Xho*I fragment ($E1_{45}$ to $E1_{192}$) or the fragment between *Sma*I and *Hin*dIII ($E1_{270}$ to $E1_{481}$) (Fig. 1).

E1 protein contains three functional N-linked glycosylation sites (Hobman *et al.,* 1991). In mutant m1, one glycosylation site is retained, while in mutants m2 and m3, all three glycosylation sites are retained. The observed apparent molecular weights of m1 (37 kDa), m2 (42 kDa), and m3 (38 kDa) suggest that they were translocated and core glycosylated, as the estimated molecular weights based on the predicted amino acid sequences for m1, m2, and m3 are 36, 33, and 28 kDa, respectively.

To determine whether if the carbohydrate moieties on E1 protein affect recognition by HI and VN MAbs, pSPT19 plasmids of p3′ E2E1, m2 and m3 were linearized with *Hin*dIII, *Bam*HI, and *Sma*I, respectively, and RNA was transcribed with SP6 RNA polymerase and translated *in vitro* using a rabbit reticulocyte system without microsomes (Hobman *et al.,* 1988). Fluorographs from translation products immunoprecipitated with MAbs are shown in Fig. 3. Mutants m2 and m3 were immunoprecipitated by both HI (3D9F) and VN (21B9H) MAbs. These results suggest that the binding capacity of both MAbs is independent of carbohydrate moieties on E1 fragments. Cell-free translation of RNAs from p3′ E2E1, m2, and m3 produced proteins with apparent molecular weights of 61, 40, and 35 kDa, respectively. The higher molecular weights observed in the translation products than predicted from amino acid sequences for m2 (33 kDa) and m3 (28 kDa) are due to the presence of eight amino acids of C protein and 69 carboxy-terminal residues of E2 in m2 and m3 which were not cleaved in the *in vitro* translation system.

To further narrow down the peptide domains containing HA and VN epitopes, N-terminal deletion mutants m4 and m5 (containing only eight amino acids of C protein, but no signal sequence of E1 were constructed (Fig. 1). We have shown previously that untranslocated E1 protein in COS cells is extremely unstable (Hobman *et al.,* 1988). Therefore, an *in vitro* translation system was used to analyze these mutants. The cDNA constructs in pSPT19 were linearized with

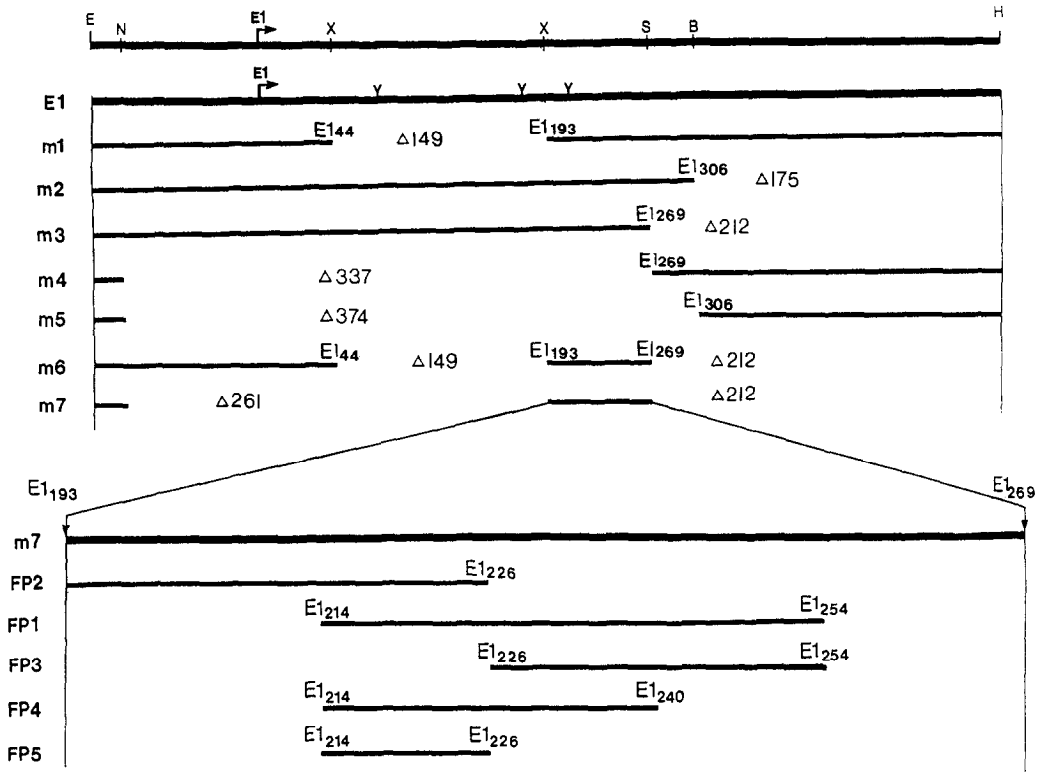


FIG. 1. Schematic representation of the cDNA fragments used for construction of E1 mutants. The deletions are denoted in terms of amino acid residues for mutants m1 to m7. The sizes of the PCR products (FP1–FP5) are also given in amino acid residues. E, *Eco*RI; N, *Nco*I; X, *Xba*I; S, *Sma*I; B, *Bam*HI; H, *Hind*III. N-linked glycosylation sites are indicated by Y. The top line shows the cDNA fragment encoding RV E1.

*Hind*III, transcribed, and translated as described (Hobman *et al.*, 1988). N-terminal-deleted mutants m4 and m5 were not immunoprecipitated by MAbs, but were immunoprecipitated by human anti-RV serum (Fig. 4). These results indicate that the HA and VN epitopes recognized by MAbs 3D9F and 21B9H are not located at the C-terminal of E1 protein and are within the fragment between *Xho*I and *Sma*I sites ($E1_{193}$ to $E1_{269}$) or the N-terminal region upstream from the first *Xho*I site ($E1_1$ to $E1_{44}$). A mutant (m6) containing both regions was therefore constructed for further epitope mapping.

## Fine mapping of HA and VN epitopes

We have attempted to express mutant m6 in COS cells, but were unable to detect m6 in immunoblots using human anti-RV serum (data not shown). As an alternative, *E. coli* pET vectors (Studier *et al.*, 1990) were used for the expression of smaller deletion mutants. Mutant cDNAs (m4 and m6) were inserted into the *Nco*I site of the pET8c vector (Rosenberg *et al.*, 1987) and expressed in *E. coli* as nonfusion proteins. The cell lysates from induced *E. coli* cultures were sep-

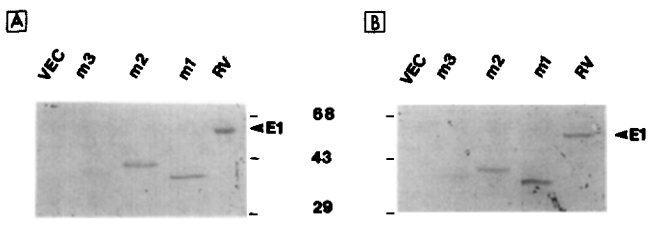


FIG. 2. Immunoblot analysis of E1 mutants expressed in COS cells. COS cells were transfected with recombinant plasmids as described under Materials and Methods. Cell lysates were separated by SDS–PAGE and transferred to nitrocellulose. E1 antigens were detected using MAbs 21B9H (A) and 3D9F (B). The relative mobilities of protein standards (kDa) are indicated. VEC, COS cells transfected with pCMV5 vector.

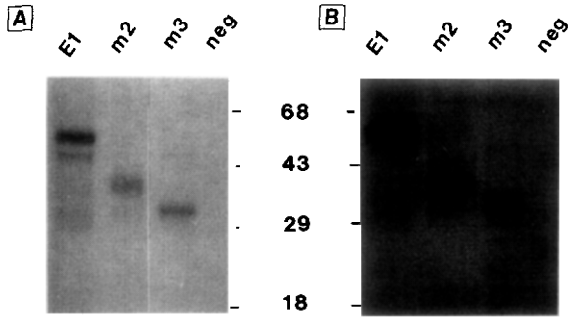


FIG. 3. *In vitro* translation of E1 mutants. The translated products were immunoprecipitated with MAbs 21B9H (A) or 3D9F (B). The positions of protein standards (kDa) are indicated.

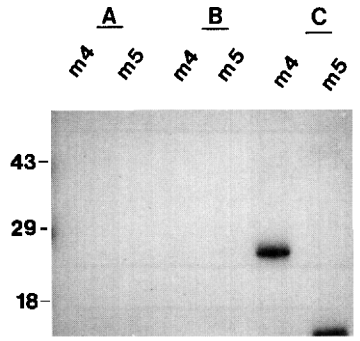


FIG. 4. *In vitro* translation of mutants m4 and m5. The translated products were immunoprecipitated with MAb 21B9H (A), MAb 3D9F (B), or human anti-RV serum (C). Protein molecular weight standards are shown on the left (kDa).

arated on a 12% SDS–PAGE and RV E1-specific polypeptides were detected by immunoblotting using MAbs (Fig. 5). Lack of recognition of m4 and positive recognition of m6 by HI and VN MAbs confirmed that HA and VN epitopes are located within these two regions ($E1_1$ to $E1_{44}$ and $E1_{193}$ to $E1_{269}$). To determine which contains HA and VN epitopes, mutant m7 was constructed (Fig. 1) and expressed in *E. coli* as a fusion protein using vector pET3xa (Studier *et al.*, 1990). Expressed fusion protein from m7 was recognized by both HI and VN MAbs (Fig. 5), suggesting that HA and VN epitopes are located within the region $E1_{193}$ to $E1_{269}$ and not in the region $E1_1$ to $E1_{44}$.

The region $E1_{193}$ to $E1_{269}$ was further divided into two smaller fragments (FP-1 and FP-2) using the polymerase chain reaction with synthetic oligonucleotides (Fig. 1). Mutants FP-1 ($E1_{214}$ to $E1_{254}$) and FP-2 ($E1_{193}$ to $E1_{226}$) were expressed as fusion proteins in *E. coli* using pET3xa vector (Studier *et al.*, 1990). Both HI and VN MAbs reacted with mutant FP-1 but not with mutant FP-2 (Fig. 5), suggesting that the corresponding epitopes are contained within the region $E1_{214}$ to $E1_{254}$ (Fig. 1). Mutant FP-1 was further subdivided into three small constructs: FP-3 ($E1_{226}$ to $E1_{254}$), FP-4 ($E1_{214}$ to $E1_{240}$), and FP-5 ($E1_{214}$ to $E1_{226}$). Binding of HI and VN MAbs by the expressed fusion proteins is shown in Fig. 5. Both HI and VN MAbs recognized mutant FP-4, but not mutants FP-3 and FP-5. The failure of FP-3 and FP-5 to react with MAbs is not due to the low levels of expression of these mutants, as abundant expressed proteins were observed in SDS–PAGE stained with Coomasie brilliant blue and were recognized by human RV polyclonal antibodies (data not shown). It is possible that the HA and VN epitopes may be interrupted by the break at the amino acids around residue $E_{226}$, or the epitopes on FP-5 and FP-3 maybe buried under the large fusion partners and inaccessible to the MAbs. Thus it was concluded from this part of the study that the epitopes defined by MAb3D9F(HI) map to a domain of 27 amino acids ($E1_{214}$ to $E1_{240}$).

## Localization of HA and VN epitopes using synthetic peptides

To define the epitopes further, six overlapping synthetic peptides (Ep11 to Ep15 and Ep25) spanning the m7 region (Fig. 6) were synthesized, purified, and coated onto ELISA plates and probed with monoclonal antibodies. Peptide-specific ELISA results were observed only with the 35 amino acid peptide EP-25 (data not shown). EP-25 was then divided into two smaller peptides, EP-24 (17 aa) and EP-26 (15 aa) (Fig. 6). Although MAb 21B9H reacted strongly with EP-25, it failed to recognize EP-24 and EP-26 in peptide-specific ELISA (Fig. 7). However, another viral neutralizing MAb (16A10E) recognized both EP-25 and EP-26 (Fig. 7), suggesting that there are two distinct viral neutralizing epitopes on E1. Three HI MAbs (3D9F, 3D5D, and 12B2D) failed to recognize any of the synthetic peptides tested (data not shown). Perhaps the hemagglutinin epitope is more dependent on the tertiary structure of the protein and hence requires a correct environment for expression of its epitope.

Combining the data obtained from the studies of the truncated forms of E1 and the peptide analyses, the results are summarized as follows:

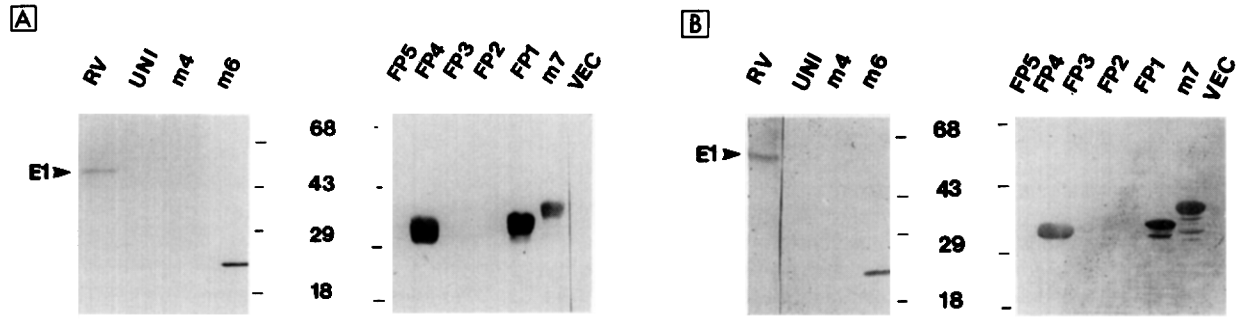


FIG. 5. Immunoblot analysis of fusion proteins expressed in *E. coli*. Blots were detected with monoclonal antibodies 21B9H (A) and 3D9F (B).

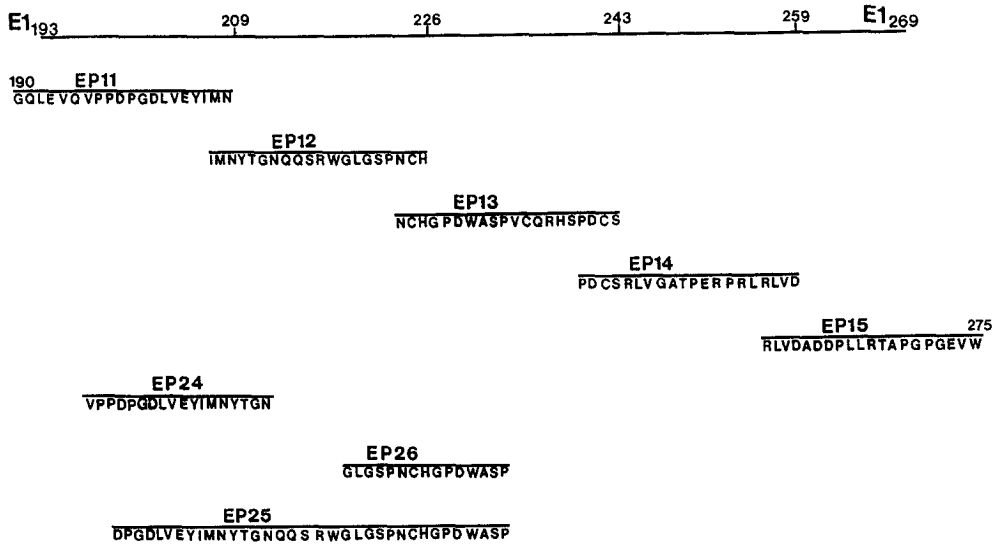


FIG. 6. Position of peptides relative to the mutant m7. The numbers indicate the positions of each peptide in E1 protein (Clarke *et al.*, 1987).

(1) The viral neutralizing epitope defined by MAb 21B9H mapped to amino acid residues 214 to 233 (QQSRWGLGSPNCHGPDWASP).

(2) The viral neutralizing epitope defined by MAb 16A10E mapped to amino acid residues 219 to 233 (GLGSPNCHGPDWASP).

(3) The hemagglutinin epitope defined by MAb 3D9F mapped to amino acid residues 214 to 240 (QQSRWGLGSPNCHGPDWASPVCQRHSP).

## DISCUSSION

Using *in vitro* and *in vivo* expression systems, 12 E1 mutants were constructed and expressed in order to identify the location of epitopes recognized by E1-specific MAbs. Due to the nature of the experiments used in this study, the epitopes that have been mapped are linear in structure and conformation-independent. Any epitopes that are dependent on native conformation may not have been located. There appears to be no general rule whether neutralizing epitopes are linear or conformational (Alexander and Elder, 1984; Long *et al.*, 1986; Wright *et al.*, 1989). For construction of synthetic peptide vaccines, it is necessary to define functional epitopes which can be mimicked by linear polypeptide fragments (Dietzschold *et al.*, 1990).

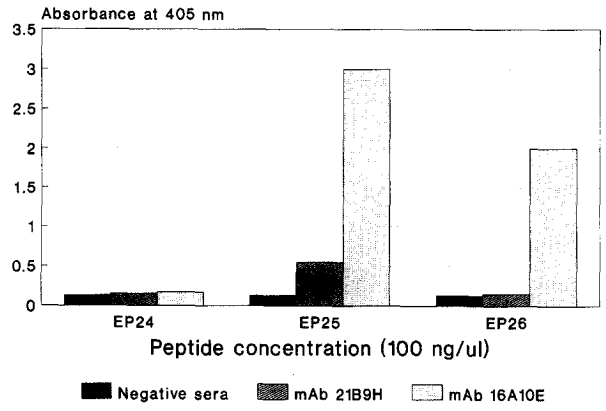


FIG. 7. Recognition of E1 peptides EP24, EP25, and EP26 by VN monoclonal antibodies 21B9H and 16A10E. 100 ng/$\mu$l of synthetic peptides were bound to Immulon-2 plates and probed with monoclonal antibodies at 1:200 dilutions. The negative sera are normal Balb/C mouse sera not exposed to rubella virus.

In general, the oligosaccharide side chains of viral glycoproteins do not act as epitopes per se, but only modulate the expression of neighboring epitopes constituted by residues of the underlying polypeptide backbone. The presence of carbohydrates preserves the conformational integrity of some epitopes that lose antigenicity upon deglycosylation (Van Regenmortel, 1990). In addition, attachment of additional oligosaccharide may prevent a monoclonal antibody from binding to its underlying epitope. However, the majority of neutralizing antibodies are not dependent on the presence of carbohydrates. Deglycosylated virus adsorbs neutralizing antibody from sera as efficiently as glycosylated virus (Van Regenmortel, 1990). For production of synthetic vaccines, epitopes that are independent of glycosylation are important since they are less likely to depend on conformation. The epitopes defined in this study are all antigenic regardless of the presence or absence of carbohydrates on E1 and its mutants.

Fig. 8 summarizes all the data obtained in this investigation. Epitopes for viral neutralizing MAbs 21B9H and 16A10E mapped to amino acid residues 214 to 233 and 219 to 233, respectively. The hemagglutinin

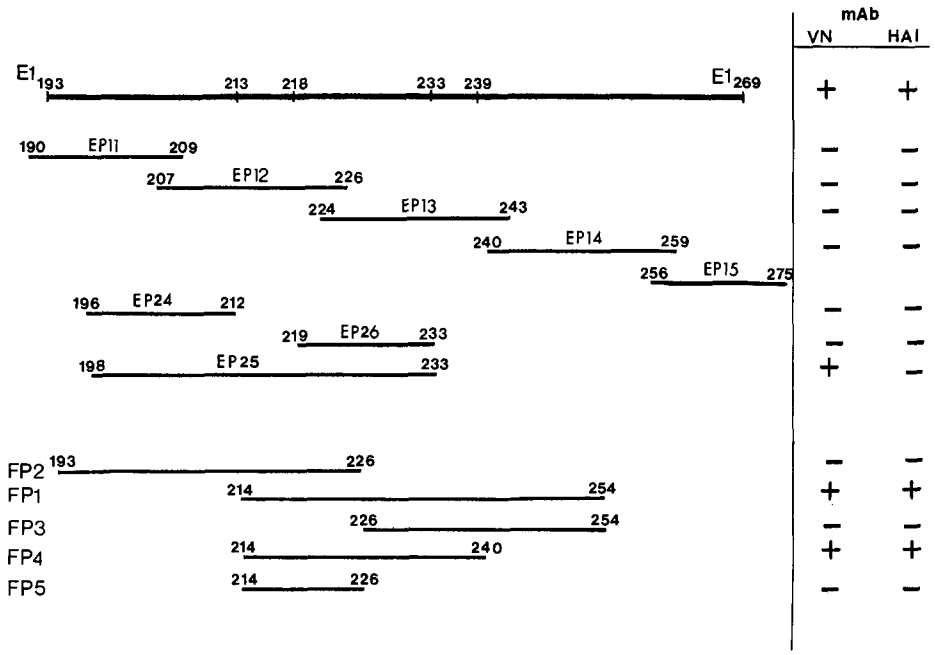


FIG. 8. Summary of the results using peptides (EP11–EP14, EP24–EP26) and PCR products expressed as fusion proteins (FP1–FP5). VN, viral neutralizing monoclonal antibody 21B9H; HI, hemagglutinin inhibiting monoclonal antibody 3D9F.

epitope defined by 3D9F mapped to amino acids 214 to 240. The inability of the monoclonal antibody 21B9H to recognize peptides EP-12, EP-13, and EP-26 as well as the expressed mutant proteins FP-2, FP-3, and FP-5 suggests that residues 214 to 219 and 226 to 233 are critical for antibody–peptide interaction. Alternatively, the epitope, upon binding to the ELISA plate, may have been altered such that the monoclonal antibody no longer recognized its epitope (Tang *et al.*, 1988). The structural data suggest that epitopes on native proteins consist of 15–20 residues with a smaller subset of 5–6 of these residues contributing most of the binding energy (Laver *et al.*, 1990). Since EP-26 is only 15 amino acids in length, it is possible that the critical 5–6 amino acid residues are not available for binding to the monoclonal antibody 21B9H following adsorption to the solid support. EP-25 (35-mer peptide) is positively recognized by the MAb 21B9H, which suggests that the epitope on this larger peptide is in the appropriate form. The surrounding extra amino acid residues may be required for appropriate presentation of the epitope for MAb 21B9H.

In contrast to MAb 21B9H, EP-26 reacted positively with MAb 16A10E. This result suggests that there are two distinct viral neutralizing epitopes close together or overlapping on a linear peptide. However MAb 16A10E failed to recognize peptides EP-12 or EP-13 and the mutant proteins FP-2, FP-3, and FP-5. This result implies that the epitope for MAb 16A10E overlaps the break regions of the above-mentioned peptides (Fig. 8) and the deletion products and is, hence, mapped to residues $E1_{219}$ to $E1_{233}$. To determine whether peptide EP-26 is the minimum VN epitope recognized by MAb 16A10E, further fine mapping is in progress.

Using various fusion protein constructs the hemagglutinin epitope as defined by MAb 3D9F mapped to FP-4 ($E1_{214}$ to $E1_{240}$). Since MAb 3D9F failed to recognize any synthetic peptides that included peptide EP-25 ($E1_{198}$ to $E1_{233}$), this implies that the epitope recognized by MAb 3D9F requires additional residues at the C-terminal of peptide EP-25. Positive recognition of FP-1 ($E1_{214}$ to $E1_{254}$) and FP-4 ($E1_{214}$ to $E1_{240}$) by MAbs further supports this conclusion. However, the absence of positive identification of EP-13 ($E1_{224}$ to $E1_{243}$) and FP-3 ($E1_{226}$ to $E1_{254}$) by the monoclonal antibody makes positive conclusion difficult. As with the epitopes for the viral neutralizing monoclonal antibodies used in this study, the hemagglutinin epitope may have been altered during the binding of the smaller peptides to the plates, resulting in negative data for EP-13. On mutant FP-3, the epitopes may not have been retained due to the fusion partner. On the other hand, proper presentation of the MAb(3D9F) epitope may require additional residues at the N-terminus of EP-13 and FP-3. Though the antibody binding residues may only be within 5–6 amino acids of FP-4, the surrounding residues may be required to maintain the stability of the antibody–antigen complex (Laver *et al.*, 1990).

Terry *et al.* (1988) have identified three epitopes

($EP_1$, $EP_2$, and $EP_3$) within the E1 region ($E1_{245}$ to $E1_{285}$). MAbs recognizing epitopes $EP_1$ and $EP_2$ show both HI and VN activities, while MAbs recognizing $EP_3$ epitope show only VN activity. Waxham and Wolinsky (1987) have defined six nonoverlapping antigenic epitopes on E1 by using MAbs in competitive inhibition studies. These workers have also mapped the HA and VN epitopes within the 82 amino acid domain of E1 ($E1_{202}$ to $E1_{283}$) using their MAbs (Wolinsky *et al.,* 1991). Since the antigenicity of an epitope recognized by MAb is dependent upon conformation of the protein, the cross-reactivity seen in MAbs probably represents binding to a proportion of denatured RV E1. It is not unexpected to observe that the epitopes mapped by our MAbs do not overlap the epitopes defined by Terry *et al.* (1988), but are adjacent to the $EP_2$ epitope, shown to have HA and VN activities. It is possible that we and Terry *et al.* have independently mapped three distinct VN epitopes on E1. The mechanisms of viral neutralization by these monoclonal antibodies are not yet clear. Neutralization by monoclonal antibodies may prevent infection directly or indirectly by binding to the glycoprotein and preventing receptor recognition or by binding to a site in proximity to the receptor binding site, causing steric hindrance or a conformational change such that the receptor-binding domain is masked or altered. Studies are in progress to determine if these HA and VN epitopes elicit any functional antibody responses.

The epitopes within the region ($E1_{214}$ to $E1_{240}$) defined in this study are consistent with the findings of Mitchell *et al.* (1992). A synthetic peptide corresponding to residues $E1_{213}$ to $E1_{239}$ was used as a target antigen in ELISA to assess the antibody responses of patients during acute and convalescent phases of wild rubella infection. It was found that the E1 peptide-reactive antibodies closely paralleled the RV-specific antibodies measured by RV ELISA, HI, and VN assays (Mitchell *et al.,* 1992). This result suggests that the epitopes defined by us may be HA and VN epitopes for human antibodies on RV E1 and may prove useful in determining effective RV immunity in diagnostic assay for rubella.

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