Mapping of the epitopes of Epstein–Barr virus gp350 using monoclonal antibodies and recombinant proteins expressed in *Escherichia coli* defines three antigenic determinants

Peng-fei Zhang,¹ Michael Klutch,¹ Gary Armstrong,¹ Louis Qualtiere,² Gary Pearson³ and Carol J. Marcus-Sekura¹

¹Division of Virology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892, U.S.A., ²Department of Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada and ³Department of Microbiology, Georgetown University, Washington D.C., U.S.A.

The Epstein–Barr virus (EBV) major surface membrane antigen (MA), gp350/220, induces antibodies that neutralize virus infectivity *in vitro*. The MA glycoprotein is encoded by nucleotides 1784 to 4504 of the *BamHI* L fragment of the EBV genome. To define the antigenic epitopes on gp350, sequences encoding portions of the protein were cloned into an *Escherichia coli* expression system and eight recombinant clones were generated, two overlapping clones representing the C terminus and six overlapping clones representing the N terminus. The epitopes expressed by the recombinant proteins were mapped using 14 anti-MA monoclonal antibodies (MAbs) in a dot blot immunoassay. One of the MAbs reacted with clones that express the C terminus of gp350 and three others reacted with clones expressing the N terminal portion of the protein; the remaining MAbs tested were not reactive with the cloned proteins. The data identify three antigenic determinants on gp350. DNA sequences encoding these epitopes are located between nucleotides 1980 and 2307, 3186 and 3528, and 3528 and 3576 of the *BamHI* L fragment. In an attempt to elicit neutralizing antibodies, rabbits were immunized with gel-purified recombinant proteins from four of the clones. Neutralization assays indicate that the proteins expressed by these clones do not induce *in vitro* virus-neutralizing antibodies.

Introduction

Epstein–Barr virus (EBV) is associated with widespread, inapparent virus infection in man and is the causative agent of infectious mononucleosis (Henle & Henle, 1979); it is also strongly implicated in the aetiology of two human neoplasias, Burkitt’s lymphoma (Epstein & Achong, 1979) and nasopharyngeal carcinoma (Klein, 1979). The development of an effective vaccine for EBV is a desirable goal. However, the use of virus-infected cells as an antigen source is problematic because the virus transforms human B lymphocytes and does not replicate well *in vitro*. Therefore, selection and production of attenuated virus for vaccine production is not feasible at the present time.

The EBV major membrane antigen (MA), gp350 [which is also present as a lower *M*₁ form, gp220, in certain strains (Biggin et al., 1984; Hummel et al., 1984; Beisel et al., 1985)], has been identified as the virus protein which interacts with the C3d cell receptor (Fingeroth et al., 1984; Nemerow et al., 1985; Wells et al., 1982), and as an inducer of specific *in vitro* virus-neutralizing antibodies (Epstein et al., 1985, 1986; Morgan et al., 1984; Qualtiere et al., 1982a; Thorley-Lawson, 1979; Thorley-Lawson & Poodry, 1982). T cell-mediated responses to gp350 have been studied by Ulaeto et al. (1988) and Bejarano et al. (1990). MA is a prime candidate for use as a recombinant vaccine because it induces neutralizing antibody, antibody-dependent cellular cytotoxicity (ADCC) (Qualtiere et al., 1982a) and cell-mediated responses.

*BamHI* clones of the entire genome of the B95-8 strain of EBV have been produced (Arrand et al., 1981), the complete nucleotide sequence of B95-8 has been determined (Baer et al., 1984), and the sequence encoding gp350 has been mapped to the BL-LF1 reading frame in the *BamHI* L fragment (Biggin et al., 1984). The availability of sequence information and the potential importance of this protein as a proposed vaccine candidate has resulted in the cloning and expression of EBV gp350 in a variety of expression systems, including *Escherichia coli* (Beisel et al., 1985), yeast (Schultz et al., 1987; Emini et al., 1988), mammalian cells (Whang et al., 1987; Emini et al., 1988) and virus vectors including
vaccinia (Mackett & Arrand, 1985) and varicella zoster viruses (Lowe et al., 1987). The immunogenicity of these recombinant proteins has been evaluated after immunization of a variety of animal species. The results have been extremely variable and this has led to the suggestion that the antigenicity of a recombinant protein is influenced significantly by the cell expressing it (Emini et al., 1988). This variability has also been attributed to the fact that the native protein is heavily glycosylated, with more than 50% of its apparent Mr being oligosaccharide (Edson & Thorley-Lawson, 1983), glycosylation by different vector systems varies both in type and extent, and to possible alterations in availability of epitopes to the immune system in different antigen preparations.

Epstein has developed an animal model for testing the ability of various vaccine preparations to prevent tumours (Epstein, 1984) and has investigated subunit preparations of gp350 purified from virions. He has found that different procedures for preparing antigen can result in either protection against virus challenge (Epstein et al., 1985) or no protection (Epstein et al., 1986), and has attributed this variability to variations in the immunogenicity of the immunogen.

It therefore appears to be essential to identify the relevant epitopes in gp350 responsible for eliciting protective immunity. We have begun to map specific epitopes on gp350 using subgenomic portions of the molecule expressed in E. coli, because this has previously proved useful in epitope mapping of human immunodeficiency virus type 1 gag proteins (Marcus-Sekura et al., 1990a). Monoclonal antibodies (MAbs) to gp350/220 have been developed and characterized (Hoffman et al., 1980; Qualtiere et al., 1982b, 1987; Thorley-Lawson & Geilinger, 1980), and assigned to different epitope groups based on competition assay results (Qualtiere et al., 1987). Some of these MAbs are neutralizing in tissue culture assays (Qualtiere et al., 1987; Emini et al., 1988) and some inhibit ADCC (Qualtiere et al., 1982a). We report the identification of three epitopes on gp350 on the basis of the reactivity of 14 MAbs with the recombinant proteins, as well as data which suggest that these epitopes do not readily elicit neutralizing antibodies.

**Methods**

**Construction of clones.** The 5053 nucleotide *Bam*HI L fragment of EBV [nucleotides 87651 to 92703 in the EBV B95–8 sequence (Baez et al., 1984; Biggin et al., 1984)] was subcloned into pBR322 from a plasmid containing EBV *Bam*HI W, w and b fragments in ψ1776, kindly provided by B. Griffin (Arrand et al., 1981). The resulting plasmid was digested with *Pst*I and *Xmn*I, resulting in the production of two fragments of similar size (nucleotides 92 to 2299 and 2230 to 4303 in *Bam*HI L). Further digestion with *Hinc*II cleaved the unwanted fragment (nucleotides 92 to 2259), allowing a 2074 bp fragment (nucleotides 2230 to 4303 in the *Bam*HI L sequence) containing the BL-LF1 open reading frame (nucleotides 4504 to 1784) encoding EBV gp350 (Biggin et al., 1984) to be purified from a 0-7% agarose gel by electroelution into a dialysis bag (Maniatis et al., 1982). A series of deletions was made by treating the 2074 bp fragment with 'slow' nuclease Bal 31 (IBI) (a species of the enzyme which is recommended for removing short stretches from duplex DNA; Maniatis et al., 1982; Wei et al., 1983) followed by treatment with T4 DNA polymerase (BRL), to blunt end the 3' termini (Maniatis et al., 1986). Insertion of a coding sequence in frame results in expression of a fusion protein when the temperature is shifted from 32 °C to 42 °C. pWS50 (Sisk et al., 1986), previously digested with *Nru*I, which recognizes a site located between the λ cII and β-galactosidase coding regions of pWS50 (Sisk et al., 1986). pWS50 contains 13 amino acids of the λ cII protein joined out-of-frame to the coding sequence of β-galactosidase, expressed under the control of the temperature-sensitive λ repressor cI857. Insertion of a coding sequence in frame results in expression of a fusion protein when the temperature is shifted from 32 °C to 42 °C. pWS60, which was used as a control plasmid, is similar to pWS50 except that the λ cII coding sequence is in frame with the β-galactosidase coding sequence, and therefore temperature shift induces expression of a λ cII–β-galactosidase fusion protein. DNA was transfected into *E. coli* DC519 (Sisk et al., 1986) using the RbCl procedure (Maniatis et al., 1982) as previously described (Marcus-Sekura et al., 1990a). Blue colonies containing EBV DNA were selected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates containing ampicillin (50 µg/ml), and further characterized by restriction enzyme digestion and nucleic acid hybridization using the Genius non-radioactive detection method (Boehringer Mannheim). Plasmid DNA was transfected into DC520 for protein expression (Sisk et al., 1986). Clones expressing different overlapping regions of EBV gp350 were identified and defined by restriction enzyme analysis and by nucleic acid sequencing of vector-insert junctions as described previously (Marcus-Sekura et al., 1990a).

Additional clones representing the C-terminal region of the gp350 amino acid sequence were generated using polymerase chain reaction (PCR) (Saiti et al., 1985; Mullis & Faloona, 1987) with a pair of primers, 5' TATACATAGTGTCGCGCTACATA 3' [nucleotides 1782 to 1807 in *Bam*HI L (Biggin et al., 1984)] and 5' CTGGAAACAGTTCCACATCCA 3' [nucleotides 2307 to 2285 on the opposite strand] being used to generate the insert. Two nucleotides (1782 and 1783, underlined), which are 3' to the coding region for gp350, were incorporated into one of the primers to put the insert in frame in pWS50. PCR was performed using 40 cycles of amplification (94 °C, 2 min; 55 °C, 2 min; 70 °C, 4 min). The amplified fragment of 526 bp (nucleotides 1782 to 2307) was purified from a 0-7% agarose gel, characterized by restriction enzyme digestion and blunt-end ligated into *Nru*I-digested pWS50. Clones were analysed by restriction enzyme digestion and sequencing of vector-insert junctions.

**Purification of recombinant protein.** Proteins were partially purified by centrifugation and urea extraction of insoluble pellets (Marcus-Sekura et al., 1990a), and used in a dot blot immunoassay or further purified by electrophoresis. For electrophoresis, urea extracts were loaded onto 6% SDS-polyacrylamide gels (1:5 mm) (Laemmli, 1970; O'Farrell & Gold, 1973) using a preparative comb. Gels were stained with Serva blue W (0.04% w/v in H2O) for 1 to 2 h and destained for 3 h in water. The fusion protein band was excised, minced and equilibrated in 40 mM-Tris-acetate pH 8.2, which was replaced three times at 15 min intervals. The gel pieces were put into an Elutrap device (Schleicher & Schuell) and protein was eluted essentially as described previously (Marcus-Sekura et al., 1990a), using 40 mM-Tris-acetate pH 8.2, 1 mM-EDTA, 5 mM-DTT, 0.05% SDS at 100 V overnight at 4 °C.
Western blot immunoassay. Protein purified by electrophoresis was loaded on precast 6% SDS–polyacrylamide gels (Schleicher & Schuell). Gels were stained with Coomassie blue or blotted onto nitrocellulose (200 mA for 2 h). Western blotting was performed as described previously (Marcus-Sekura et al., 1990b).

Preparation of B95-8 and BJAB cell lysates. B95-8, a marmoset cell line transformed with EBV which expresses transforming virus (Miller & Lipman, 1973) was grown in RPMI 1640 containing 10% heat-inactivated foetal bovine serum (FBS; Gibco), 2 mM-glutamine, 100 units of penicillin and 100 µg/ml streptomycin, and stimulated with 20 ng/ml 12-O-tetradecanoylphorbol 13-acetate and 3 mM-sodium butyrate for 72 h at 37 °C. BJAB, an EBV-negative lymphoma cell line (Clements et al., 1975), was grown under identical conditions, except 20% heat-inactivated FBS was used. Cells were washed twice with PBS pH 7-2 and pelleted at 1700 r.p.m. ( Sorvall RT6000B) for 10 min at 10 °C. Pelleted cells (1 ml) were lysed by the addition of 3 ml of lysis buffer (0·02 M-Tris–HCl pH 7·4, 0·15 M-NaCl, 0·016 M-PMSF, 0·12 M-sodium deoxycholate, 0·007% 2-mercaptoethanol, 0·5% NP40) and sonicated (Branson model W-140, output 5) on ice with five pulses of 20 s each. After centrifugation at 27000 g for 30 min at 4 °C, the supernatant was used in a dot blot immunoassay.

Dot blot immunoassay. Recombinant proteins prepared by urea extraction of the E. coli clones and B95-8 cell lysates containing virus-produced gp350 (A280, 0·7-1·2) were diluted in PBS to the same concentration (A280, 0·5), and 200 µl per well was loaded using gentle suction onto a dot blot apparatus (Schleicher & Schuell) containing a nitrocellulose membrane previously equilibrated with PBS pH 7-2. In the apparatus, the nitrocellulose was dried at room temperature, washed once with PBS pH 7-2 and blocked with 5% non-fat milk in PBS pH 7·2 for 2 h. The nitrocellulose membrane was then removed from the apparatus and washed five times with PBS–TWEEN 20, incubated with biotinylated anti-mouse IgG (Vector) (diluted 1:1000) for 1 h at 37 °C, washed five times with PBS–TWEEN 20, incubated with horseradish peroxidase-avidin D (Vector) diluted 1:1000 for 1 h at 37 °C, washed five times with PBS–TWEEN 20, and colour was developed with 4-chloro-l-napthol and H2O2 (Marcus-Sekura et al., 1988).

Production of monospecific antibody in rabbits. Recombinant proteins from E4, E201, E70, E203 and pWS60, as a control, were purified by electrophoresis, and aliquots of 250 µg were emulsified in complete Freund's adjuvant and injected into rabbits interdermally (0·05 ml, five sites) and subcutaneously (0·2 ml, two sites). Booster inoculations (250 µg) in incomplete Freund's adjuvant were administered intradermally on day 30. Sera were obtained by bleeding 2 weeks and 4 weeks after primary inoculation, and 2 weeks after the boost. Sera were tested by dot blot immunoassay and immunofluorescence assay (IFA) (Henle & Henle, 1966).

Neutralization assay. For the assay using human cord blood lymphocytes, sera and MAbs were heat-inactivated at 56 °C for 30 min. To obtain virus, B95-8 cells were aged at high density for 10 days at 37 °C and the supernatant was collected, clarified by low speed centrifugation and double-filtered through a 0·45 µm filter. The sera and MAbs to be tested were diluted in PBS pH 7·2. EBV B95-8 with a titre of 10⁵ transforming units/ml was used. B95-8 virus (0·5 ml) was combined with 0·5 ml of diluted serum and incubated for 1 h at 37 °C, after which the combined virus–serum mixture was inoculated onto 3 x 10⁶ human cord blood lymphocytes and adsorbed for 2 h at 37 °C. After adsorption the cells were washed three times with RPMI 1640 and resuspended in RPMI 1640 containing 20% FBS. Incubation was continued until the cord blood cells either died or were immortalized (6 to 8 weeks), as determined by continuous growth and the presence of EBV nuclear antigen (EBNA), assayed by IFA.

For the assay using Raji cells, sera were heat-inactivated at 56 °C for 30 min. Early antigen (EA)-inducing virus was obtained from the P31B cell line, a clone of P3HR1 which produces high levels of EA and viral capsid antigen (VCA) (G. Armstrong, unpublished data). Cells were aged at high density for 10 days at 33 °C, and supernatant was harvested, clarified by low speed centrifugation at 10 °C, double-filtered through a 0·45 µm filter and kept on ice. Sera were diluted 1:5 in PBS pH 7·2 and 0·5 ml of serum was combined with 0·5 ml virus and incubated at 37 °C for 1 h. The combined virus–serum mixture was inoculated onto Raji cells (Epstein et al., 1976) (3 x 10⁶) and adsorbed at 37 °C for 2 h. After adsorption, the cells were washed three times with PBS pH 7·2 and resuspended in RPMI 1640 containing 10% heat-inactivated FBS. Cells were harvested after 48 h and prepared for IFA. MAbs D-R3, R-R63 and V-L2 to EA(D), EA(R) and VCA, respectively, were used in IFA at 1:100 dilution. Sera were considered to be positive for neutralization if EA production was inhibited compared to negative control sera.

Results and Discussion

Description of subgenomic clones of EBV MA used for epitope analysis

To define B cell epitopes on EBV gp350, a series of clones expressing overlapping portions of the amino acid sequence of gp350 was generated. The temperature-inducible plasmid expression vector pWS50, which contains the λ cII nucleotide sequence out-of-frame with the β-galactosidase gene (Sisk et al., 1986), was used to express the clones. The plasmid was digested at an NruI site located between the λ cII and β-galactosidase sequences, and coding sequences of gp350 were inserted so that the EBV sequence was in frame with both the 5’ λ cII nucleotide sequence and the 3’ β-galactosidase sequence. This resulted in the expression of tripartite fusion proteins containing 13 amino acids of λ cII at the N terminus, followed by a portion of EBV gp350 fused to β-galactosidase at the C terminus; colonies expressing the protein were selected on X-gal plates. Six overlapping clones representing the N terminus of gp350 were generated by Bal 31 and T4 polymerase treatment of the PsuII–XmnI fragment (nucleotides 2230 to 4303) of EBV BamHI L (clone E203, nucleotides 2301 to 4287; E204, 2625 to 4266; E7, 3186 to 4266; E70, 3069 to 4146; E109, 3528 to 4266; E201, 3576 to 4266). All of these clones contain nucleic acid sequences located at the 5’ end and in the central portion of the coding sequence of EBV gp350.

To obtain clones from the 3’ end of the coding sequence of gp350, the 3’ sequence containing nucleotides 1782 to 2307 was constructed using PCR, and two overlapping clones (E4, nucleotides 1782 to 2307; E9,
1980 to 2307) representing the C terminus of gp350 were isolated. Thus, the eight overlapping clones contain nucleotide sequences representing most (92%) of the coding sequence of gp350, with the exception of 217 bp (nucleotides 4288 to 4504). Of these 217 bp, the N-terminal 54 are thought to encode an 18 amino acid, cleavable signal peptide (Whang et al., 1987) and are therefore not useful in defining biologically important epitopes.

Characterization of the recombinant antigens with anti-EBV antiserum

Recombinant proteins expressed in *E. coli* by the eight different clones were induced by temperature shift, purified, and characterized by SDS-PAGE and Western blot analysis. The eight proteins containing portions of gp350 migrated as distinct entities on 6% SDS-polyacrylamide gels as shown in Fig. 1(a). The proteins migrated as predicted, in proportion to the size of the EBV insert in the tripartite fusion protein, the control containing no EBV insert migrating fastest. Apparent *M*<sub>r</sub>s of the recombinant proteins calculated using linear regression analysis based on migration of the *M*<sub>r</sub> markers were 120K for the λ cII-β-galactosidase fusion protein, 127-7K (E9), 135K (E4), 139K (E201), 143K (E109), 151K (E70), 160K (E7), 184-6K (E204) and 189-9K (E203). This is close to the predicted *M*<sub>r</sub>s, calculated from the number of amino acids (using 0.1K per amino acid), of 116K, 127K, 133K, 139K, 141K, 151K, 152K, 171K and 182K, respectively.

The results of Western blot analysis of the recombinant proteins expressed by the eight clones with MAb against β-galactosidase and anti-EBV-positive human serum are shown in Fig. 1(b and c). All the recombinant proteins expressed by clones containing EBV gp350 sequences reacted with both MAb against β-galactosidase (Fig. 1b, lanes 2 to 9) and anti-EBV-positive human serum (Fig. 1c, lanes 2 to 9), indicating that they are fusion proteins of β-galactosidase and EBV gp350, and that all contain epitopes recognized by human sera. Clones E70, E7, E204 and E203, containing larger fragments of the gp350 gene (Fig. 1c, lanes 6 to 9), expressed proteins which had stronger reactivity with the human anti-EBV-positive serum than clones E9, E4, E201 and E109, containing smaller gene fragments (c, lanes 2 to 5). This is of interest because there was apparently less protein present in the two largest protein bands from E204 and E203, as demonstrated by less staining with Coomassie blue (a, lanes 8 and 9) and less reactivity with MAb against β-galactosidase (b, lanes 8 and 9). This increased reactivity of the larger recombinant proteins with anti-EBV-positive human serum may be due to the presence of additional epitopes reactive with the human antiserum in the larger proteins, or the presence of one or more highly antigenic regions expressed by the larger clones and absent in the smaller clones. Fig. 1(b) contains some material of lower apparent *M*<sub>r</sub>, than the major protein bands reactive with antibody to β-galactosidase, suggesting the possibility of smaller species arising by either reinitiation of translation, which is relatively common in *E. coli*, or some protein degradation. The absence of similar reactivity with EBV-specific antiserum in the region below the major band in Fig. 1(c) suggests that reinitiation may be more probable. The protein expressed by pWS60 was only reactive with the MAb against β-galactosidase (Fig. 1 b, lane 1) and unreactive with the anti-EBV-positive human serum, as was expected (Fig. 1 c, lane 1). All of the
Epitope mapping of EBV gp350

Fig. 2. Epitope mapping by dot blot immunoassay. Urea extracts containing recombinant proteins (200 µl per well) produced by the eight clones and a control (columns 1 to 9) and cell lysates (columns 10 to 11) were spotted onto nitrocellulose vertically. After blocking of the membrane with non-fat milk, MAbs raised against EBV gp350/220 were applied horizontally (rows A to F). The membrane was then incubated with biotinylated anti-mouse IgG, followed by incubation with horseradish peroxidase-avidin-D and colour development with 4-chloro-l-naphthol and H₂O₂. Columns 1 to 11, E203, E204, E7, E70, E109, E201, E4, E9, pWS60, B95-8 and BJAB. Rows A to F, F29-89-A7, F29-89-D8, F34-2B11, F34-5H7, BMA-17 and 14C4.

recombinant fusion proteins also reacted with an MAb specific for the λ cII region (Zweig et al., 1987) (data not shown). No reactivity was seen when Western blot analysis was performed using an anti-EBV-negative human serum (data not shown).

Analysis of reactivity of the recombinant proteins with gp350-specific MAbs

The reactivity of 14 MAbs against B95-8 gp350 with the recombinant proteins expressed by the eight clones was studied using a dot blot assay in which cloned protein was spotted in columns onto nitrocellulose and the different MAbs were applied horizontally in rows. Four of the MAbs tested reacted with the clones (Fig. 2). Two subclones of one MAb, F29-89-A7 and F29-89-D8 (Fig. 2, rows A and B), recognized the proteins expressed by E4 and E9; two other MAbs, F34-2B11 and F34-5H7 (rows C and D), recognized the proteins expressed by E203, E204, E7 and E70; one MAb (BMA 17; row E) recognized the proteins expressed by E203, E204, E7, E70 and E109. The protein expressed by E201 was not recognized by any of the MAbs. An MAb to poliovirus, 14C4 (row F), used as a negative control, showed no reactivity with any of the cloned antigens or with the B95-8 positive control antigen. All MAbs reactive with the recombinant proteins showed no reactivity with pWS60 (a control vector expressing λ cII–β-galactosidase fusion protein only; column 9). All reactive MAbs reacted positively with B95-8 cell lysate (EBV-producer cell line; column 10) and had no reactivity with BJAB cell lysate (EBV-negative cell line; column 11). The remaining 10 MAbs showed no reactivity with any of the cloned or pWS60 antigens, or with BJAB cell lysates, but reacted with the control EBV gp350 antigen isolated from B95-8 (data not shown). This reactivity with the antigen from B95-8 suggests that the assay was effective in detecting the reactivity of all 14 MAbs, and that the lack of reactivity of 10 of the MAbs with the recombinant antigens was probably due to absence of the appropriate epitopes in a recognizable form. Analysis by Western blotting was also attempted; however, the MAb reactivity was variable, sensitivity was poor and some MAbs did not react with the B95-8 control cell lysate.

Assignment of epitopes

Since each of the five MAbs was reactive with more than one recombinant protein containing overlapping amino acid sequences, further analysis allows the coding sequences of gp350 common to the set of reactive clones to be identified. Those sequences containing the epitopes recognized by the MAbs are shown in Fig. 3 and 4. Subclones F29-89-A7 and F29-89-D8 of F29-89 reacted with the proteins expressed by E4 and E9 only. Since the entire E9 sequence is contained within E4 (Fig. 3), the epitope recognized by this MAb (termed epitope I) is encoded between nucleotides 2307 and 1980, which corresponds to amino acids 733 to 841 of gp350 (Fig. 4). [The numbering of all amino acids has been corrected for two nucleotides at the 5' terminus, which are required to place the sequence in frame with λ cII, and two nucleotides at the 3' end which are required to put the sequence in frame with β-galactosidase.] Interestingly, this epitope is immediately upstream of the putative hydrophobic transmembrane region located near the C terminus (Beisel et al., 1985), which corresponds to amino acids 861 to 888. Furthermore, this epitope corresponds to epitope group IV defined by Qualtiere et al. (1987).

Gp220 is derived from gp350 by one internal splice, which maintains the reading frame but deletes amino acids 501 to 749. Since a portion of the region spliced out in gp220 is contained within the epitope recognized by F29-89 (amino acids 733 to 841) studies are in progress to characterize this epitope further by investigating the ability of these MAbs to react with gp220.

Two additional MAbs, F34-5H7 and F34-2B11, reacted with four recombinant proteins only (those from E70, E7, E204 and E203). The nucleotide sequence common to the four clones extends from the 5' terminus of E70, at nucleotide 4146, to the 3' terminus of E7, at nucleotide 3186 (Fig. 3). Since these MAbs did not react with E109 sequences 5' to nucleotide 3528 do not appear to encode this epitope. Epitope II is therefore encoded...
Fig. 3. Location of the three antigenic determinants within the eight clones used for epitope mapping. The top portion illustrates the 5053 nucleotide BamHI L fragment of EBV B95-8 (nucleotides 87651 to 92703) which encodes the membrane glycoprotein gp350 in the leftward reading frame BL-LF1 (nucleotides 4504 to 1784). The different hatched regions within the gp350 coding region represent epitopes I (2307 to 1980), II (3528 to 3186) and III (3576 to 3528). The eight overlapping clones (E9, E4, E201, E109, E70, E7, E204 and E203) are shown below; vertical dotted lines define the presence or absence of a particular epitope in a clone.

between nucleotides 3186 and 3528 (Fig. 3), which correspond to amino acids 326 to 439 of gp350. We cannot, however, entirely rule out the possibility that epitope II may extend beyond nucleotide 3528 toward 3576, because all four reactive clones contain these nucleotides, and this sequence or a portion of it might be necessary, but not sufficient for recognition. In addition, it is of interest that both F34-5H7 and F34-2B11 have previously been ascribed to the same epitope group (group VI) in competition studies (Qualtiere et al., 1987).

The remaining MAb, BMA 17, was reactive with the four antigens recognized by F34-5H7 and F34-2B11, but was also reactive with the protein expressed by E109. This maps the sequence encoding the epitope recognized by this MAb to between nucleotides 3528 (the C terminus of E109) and 4146 (the N terminus of E70).

Since BMA 17 was not reactive with the protein expressed by E201, epitope III can be more finely mapped to nucleotides between 3576 (the C terminus of E201) and 3528 (the C terminus of E109) (Fig. 3); nucleotides between 3530 and 3574 encode 15 amino acids which define epitope III (amino acids 310 to 325) (Fig. 4).

One possible explanation for the lack of reactivity of 10 of the 14 MAbs tested with the E. coli-expressed proteins is that these proteins are not glycosylated; alternatively the epitopes may be conformation-dependent. All the MAbs, except BMA 17, were raised against purified virus or whole cell preparations after induction (Qualtiere et al., 1987). BMA 17 was raised (G. Pearson, unpublished data) against a bacterially expressed, unglycosylated membrane antigen, III57 encoded by nucleotides 3611 to 2083 in BamHI L (Beisel et al., 1985), and is directed against a glycosylation-independent site (Emini et al., 1988). The epitope we have defined is located within the coding sequence of the expressed antigen used to raise BMA 17 as expected. The MAb 2F5.6 has been shown to react with gp350, but not gp220 (Qualtiere et al., 1982b), which is identical to gp350 except that it contains an internal splice (nucleotides 2257 to 3003). The entire sequence spliced out of gp220 is contained in clone E203 and, consistent with the explanation that glycosylation or other secondary modifications not present in E. coli-produced proteins are required for MAb recognition, the protein produced by this clone failed to react with 2F5.6. It should also be
pointed out that these three epitopes were identified using mouse MAbs and, because the pattern and quality of B cell epitope recognition may be species-specific, further experiments are needed to determine whether these epitopes elicit human antibody production.

**Examination of the ability of recombinant antigens to elicit in vitro EBV-neutralizing antibodies**

To investigate whether the recombinant proteins, which together encompass 92% of the gp350 amino acid sequence, contain functional neutralizing epitopes, proteins from four clones (E4, E201, E70 and E203 which contain all the amino acid sequences contained within the eight clones) and control antigen from pWS60 were used to immunize rabbits. Rabbit sera raised against the cloned EBV antigens, but not pWS60, reacted with gp350 on the plasma membrane of B95-8 cells in an indirect IFA (data not shown). The rabbit sera were also reactive at dilutions up to 1:1000 with the recombinant proteins expressed by the homologous clones, and at dilutions up to 1:100 with B95-8 cell lysates, in a dot blot immunoassay (data not shown). Initially, rabbit sera pre- and post-immunization with E4- or E70-encoded antigens were examined for their ability to neutralize EBV using an assay employing cord blood lymphocytes (Table 1a). F29-167-A10, an MAb previously shown to be capable of neutralizing EBV in a tissue culture assay in the absence of complement (Qualtiere et al., 1987), was used as a positive control. A second MAb, F29-89, which demonstrated equivocal ability to react in tissue culture (Qualtiere et al., 1987; Emimi et al., 1988) but did react with recombinant antigens from E4 and E9, was also tested. Both the EBV-neutralizing human serum and F29-167-A10 neutralized EBV in the assay, as evidenced by the inhibition of transformation and lack of EBNA production detectable by immunofluorescence. F29-89 (subclone F29-89-A7), F16-11B7 and the rabbit sera obtained before and after immunization with the recombinant antigens failed to neutralize EBV as evidenced by the occurrence of both transformation and the presence of EBNA in samples treated with these antibodies.

A second neutralization assay was performed using Raji cells and serum from rabbits, pre- and post-immunization with each of four recombinant proteins and protein from bacteria containing pWS60 (Table 1b). EBV neutralization-positive and -negative human sera were used as controls. MAbs detecting EBV EA(D) and EA(R) or VCA were used in IFA to detect the presence of EBV antigen; an MAb against poliovirus (Singer et al., 1989) was used as a negative control. None of the sera except the anti-EBV-positive human serum was able to inhibit production of EBV EAs in this assay, indicating that none of the recombinant antigens elicited EBV-neutralizing antibodies under these conditions. As expected, VCA was not detected because Raji cells can not be induced to produce VCA.

These results indicate that the clones do not express antigen in a form which can readily elicit virus-neutralizing antibodies. This is consistent with the absence of reactivity of any of the cloned antigens with any of the MAbs in group I (Qualtiere et al., 1987) tested (F30-3C2-C11, F34-6B5-A2, F34-1F2 and F29-167-A10). It has been suggested that group I recognizes the major neutralizing epitope of gp350. This observation is supported further by results of a previous study which used a fusion protein containing a partial sequence of EBV gp350 expressed in E. coli as an immunogen (III57 containing nucleotides 3611 to 2083; Beisel et al., 1985). Even after repeated immunization (12 inoculations of 500 µg each over a period of 8 months) this protein induced only a weak neutralizing antibody response.
Another study (Emiri et al., 1988) showed that yeast-derived EBV MA was not capable of eliciting virus-neutralizing antibody; however, several mammalian cell-derived gp350 recombinant proteins from rodent and primate cells were capable of inducing EBV-specific neutralizing antibodies in mice (Whang et al., 1987).

Since proteins produced in E. coli are not glycosylated or otherwise post-translationally modified and may not be properly folded, and proteins produced in yeast are glycosylated differently from those made in the mammalian cell, these results suggest that the epitope(s) on gp350 responsible for inducing neutralizing antibody may need to be properly glycosylated or may require a specific conformation, or both; other post-translational modifications may play a role. We cannot exclude the possibility that by using a prolonged immunization schedule such as that described by Beisel et al. (1985) some weak neutralizing activity might be observed. We can conclude, however, as others have observed, that E. coli-expressed gp350 does not readily elicit neutralizing antibodies and does not react with MAbs shown to possess neutralizing activity. We are currently expressing subgenomic portions of gp350 in a vector system which glycosylates the protein to define additional epitopes recognized by these MAbs and to pinpoint the neutralizing epitope(s).

References


Epitope mapping of EBV gp350


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