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Mapping of a region of dengue virus type-2 glycoprotein required for binding by a neutralizing monoclonal antibody

(Recombinant DNA; λ p_L promoter; expression in *Escherichia coli*; epitope mapping; PCR; deletion mutagenesis; mAb; synthetic peptide antigen)

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SUMMARY

Envelope glycoprotein E of flaviviruses is exposed at the surface of the virion, and is responsible for eliciting a neutralizing antibody (Ab) response, as well as protective immunity in the host. In this report, we describe a method for the fine mapping of a linear sequence of the E protein of dengue virus type-2 (DEN-2), recognized by a type-specific and neutralizing monoclonal Ab (mAb), 3H5. First, an *Escherichia coli* expression vector containing a heat-inducible λ p_L promoter was used to synthesize several truncated, and near-full length E polypeptides. Reactivities of these polypeptides with polyclonal mouse hyperimmune sera, as well as the 3H5 mAb revealed the location of the 3H5-binding site to be within a region of 166 amino acids (aa) between aa 255 and 422. For fine mapping, a series of targeted deletions were made in-frame within this region using the polymerase chain reaction (PCR). The hydrophilicity pattern of this region was used as a guide to systematically delete the regions encoding the various groups of surface aa residues within the context of a near-full-length E polypeptide by using PCR. The 3H5-binding site was thus precisely mapped to a region encoding 12 aa (between aa 386 and 397). A synthetic peptide containing this sequence was able to bind to the 3H5 mAb specifically, as shown by enzyme-linked immunosorbent assay. In addition, we show that rabbit Abs raised against the synthetic peptide of 12 aa were able to bind to the authentic E protein, and to neutralize DEN-2 virus in a plaque reduction assay.

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Abbreviations: *A*, absorbance; aa, amino acid(s); Ap, ampicillin; bp, base pair(s); C, capsid protein; cDNA, DNA complementary to RNA; DEN-2, dengue virus type 2; DMEM, Dulbecco's modified Eagle's medium; DOC, deoxycholate; E, envelope protein; ELISA, enzyme-linked immunosorbent assay; HI, hemagglutination-inhibition; HMAF, hyperimmune mouse ascitic fluid containing polyclonal antibodies against a mixture of DEN-2 antigens; HPLC, high-performance liquid chromatography; HSV-2, herpes simplex virus type 2; JE, Japanese encephalitis virus; kb, kilobase(s) or 1000 bp; KLH, keyhole limpet hemocyanin; LB, Luria-Bertani (medium); M, membrane protein; mAb, monoclonal antibody; MBS, *m*-maleimidobenzoylsulfosuccinimide ester; m.o.i., multiplicity of infection; NGS-C, New Guinea C strain; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; PBS, phosphate-buffered saline (10 mM Na·phosphate, pH 7.2/150 mM NaCl); PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; PCR, polymerase chain reaction; pfu, plaque forming units; PMSF, phenylmethylsulfonyl fluoride; prM, precursor to membrane protein; PRNT, plaque reduction neutralization test; RBS, ribosome binding site; SDS, sodium dodecyl sulfate; *Taq*, *Thermus aquaticus* YT1; TBE, tick-borne encephalitis; WN, West Nile virus; YF, yellow fever virus.

INTRODUCTION

Dengue is a human disease caused by dengue virus (DEN), a member of the family flaviviridae (Westaway et al., 1985). The viral genome is a single-stranded RNA of 10723 nt (New Guinea C strain) in length, having a type-1 cap at the 5′ end, but lacking a poly(A) tract at the 3′ end (for a review, see Chambers 1990). The genomic RNA is of positive-strand polarity having a single ORF that encodes a polyprotein of 3391 aa in length, which is then processed into three structural and at least seven nonstructural proteins so far identified. The four distinct serotypes of DEN are transmitted to humans principally by *Aedes aegypti* mosquitos.

The three structural proteins are the C, prM and E in mature flavivirus virion (Stollar, 1969), and the E protein has been shown to exhibit multiple biological activities. It is the major target for the induction of neutralizing antibodies, and has the property of inducing a protective immune response in the host (Buckley and Gould, 1985; Della-Porta and Westaway, 1977; Kitano et al., 1974; Heinz et al., 1981; Monath et al., 1984). It can also passively protect mice in vivo against lethal intracerebral DEN-2 challenge (Kaufman et al., 1987). The E protein is

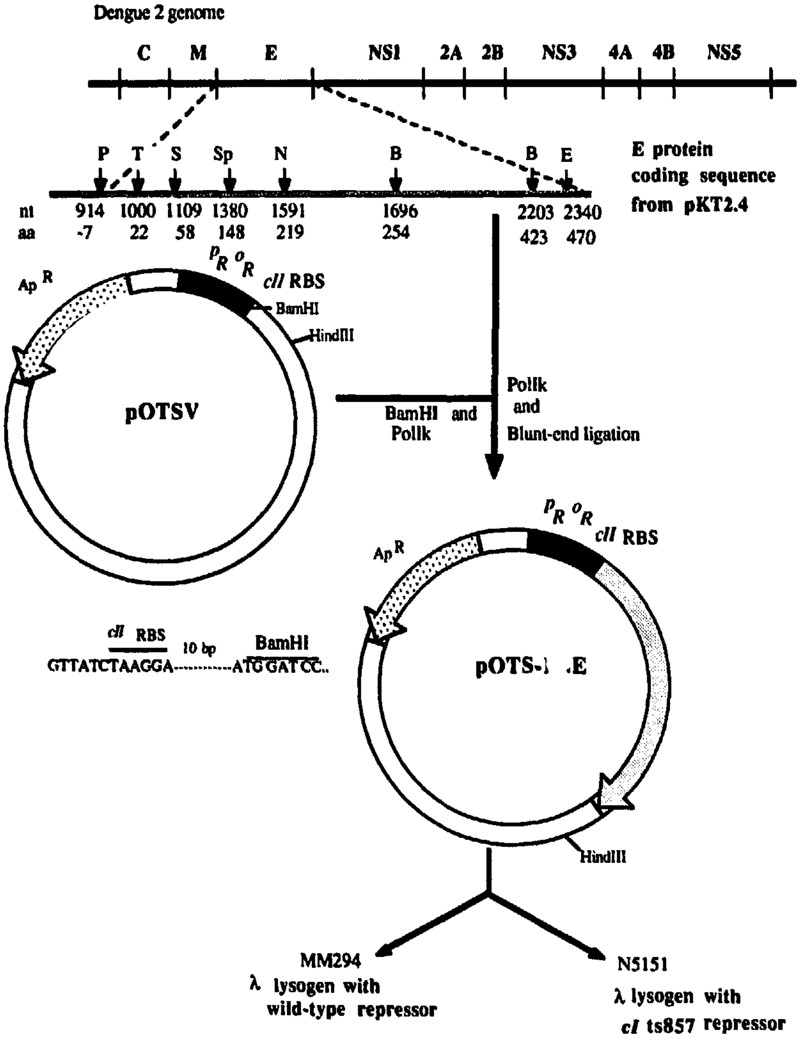

Fig. 1. Plasmid constructs for the expression of DEN-2 E polypeptides. DEN-2 polyprotein precursor encoded from a single ORF is shown in the top line. The cDNA clone pKT 2.4 (Irie et al., 1989) was digested with B, *Bam*HI; E, *Eco*RI; N, *Nco*I (partial); P, *Pvu*II; S, *Sca*I; Sp, *Sph*I; or T, *Tth*111I, and the fragments were made blunt by treatment with PolIk in each case. These fragments were cloned into the pOTS vector (pOTSV) plasmid [containing λ phage left operator and promoter (o_Lp_L), and a CII ribosome-binding site (*cII* RBS)], which was digested with *Bam*HI and treated with PolIk prior to cloning using standard techniques (Maniatis et al., 1982). The expression plasmids of general structure pOTS-D2E were obtained in which the size of the DEN-2 E coding sequence varied as follows: pTT10 (*Pvu*II-*Eco*RI), pTT15 (*Tth*111I-*Eco*RI), pTT20 (*Sca*I-*Eco*RI), pTT25 (*Sph*I-*Eco*RI), pTT30 (*Nco*I-*Eco*RI), and pTT35 (deletion of nt 1697–2203 between the two *Bam*HI sites). In all cases, the reading frame for the expression of the corresponding E polypeptide was kept in-frame and was verified by the nt sequence analysis. For transformation, and initial screening of the proper orientation of the cDNA insert, *E. coli* MM 294(λcI^+) was used, and for expression N5151(λcIts857) was used.

involved in binding of the virus to the host cell, and is also the antigen responsible for hemagglutination of erythrocytes at acid pH (Sweet and Sabin, 1954). Similar to the membrane glycoproteins of other enveloped viruses, the flavivirus E protein is quite variable at least in certain antigenically active domains (Heinz, 1986). The variable portion of the E protein contains epitopes which determine the type specificity and complex specificity. These antigenic specificities form the basis for serological classification of flaviviruses as determined by HI and neutralization tests. Identification of the neutralizing epitopes of the E protein is important to understand the mechanism of virus-host cell interaction, and to develop an effective vaccine to control the dengue virus infections.

Various methods are available to determine the precise location of antigenic sites on a protein molecule (Benjamin et al., 1984; Berzofsky, 1985). One approach involves the use of mAbs as specific reagents for defining single epitopes on the complex antigenic structure of protein molecules. Using methods involving screening of purified proteolytic fragments of a protein molecule and/or screening a collection of overlapping synthetic peptides, the antigenic determinants of a number of viral glycoproteins have been studied (Emini et al., 1982; Lubeck and Gerhard, 1982; Massey and Schochetman, 1981; Mehra et al., 1986; Roehrig et al., 1982; Volk et al., 1982). Using RNA recombination as a genetic tool, the antigenic determinants involved in neutralization, as well as virus neuropathogenicity of the peplomer protein encoded by gene *C* of murine coronavirus were localized at the C-terminal one third of the peplomer (Makino et al., 1987). Lobigs et al. (1987) defined the antigenic determinant involved in the neutralization of YF on the N-terminal region of the E protein, which is hydrophilic, rich in Cys, and not conserved among flavivirus subgroups. The approach used was to select neutralization escape variants against two neutralizing mAbs, and sequence the variants in the region of the genome encoding the E and M proteins. It was found that each of the variants resulted from a single base change in the E protein coding sequence leading to a nonconservative substitution at aa^{71} or aa^{72}. An alternative method involves the construction of expression libraries using the λgt11 system and fragments of gene encoding the antigen, and sequence all the cDNA expression clones that reacted with a specific mAb. The DNA sequence encoding the epitope is attributed to sequences that are shared by multiple antibody-positive recombinant clones (Mehra et al., 1986).

The aim of the present study was to map the antigenic determinant of the DEN-2 E protein recognized by the 3H5, a type-specific mAb, which was shown to have a significantly high neutralization titer (Gentry et al., 1982; Henchal et al., 1985). The expression system used by us for mapping the 3H5-binding site on the E protein significantly differs from the previously reported method (Mehra et al., 1986) in that in our system the polypeptides produced in *E. coli* cells are not fused to any bacterial protein.

RESULTS AND DISCUSSION

(a) Synthesis of the E protein in *Escherichia coli*

In order to map the neutralizing determinant of DEN-2 E recognized by the 3H5 mAb, we have made use of an *E. coli* expression vector pOTS (Fig. 1). It contains a unique

TABLE I

Map positions of E polypeptides encoded by expression clones

Restriction fragment of cDNA used for cloning[a]	Recombinant expression clone	Amino acid coordinates of E polypeptide	Size of polypeptides in SDS/PAGE (kDa)
*Pvu*II-*Eco*RI (nt 914–2340)	pTT10	aa −8[b]–470[c]	<48[d]
*Tth*111I-*Eco*RI (nt 1000–2340)	pTT15	aa 22–470	48
*Sca*I-*Eco*RI (nt 1109–2340)	pTT20	aa 58–470	41
*Sph*I-*Eco*RI (nt 1380–2340)	pTT25	aa 48–470	32
*Nco*I-*Eco*RI (nt 1591–2340)	pTT30	aa 219–470	25
Deletion of *Bam*HI fragment (Δ nt 1696–2203) from pTT15	pTT35	aa 22–254 fused with aa 423–470	36

[a] Numbers (nt) correspond to the DEN-2 RNA (NGS-C strain) reported by Irie et al. (1989). Δ = deletion.
[b] A minus sign denotes the position upstream of the authentic N terminus of E protein.
[c] The wild type E protein is 495 aa in length.
[d] The size of the polypeptide could not be detemined accurately due to its degradation.

*Bam*HI site for cloning foreign genes downstream from the λ p_L promoter, the ribosome-binding site, and the translation-initiation site (Rosenberg et al., 1983). For expression, the cDNA fragment encoding the E polypeptide between *Pvu*II and *Eco*RI sites of the pKT2.4 plasmid (Irie et al., 1989) was cloned into the pOTS vector (pTT10 in Table I) was used. The polypeptide encoded by pTT10 contains the E sequence from aa 1 to aa 470, as well as seven hydrophobic aa upstream from the N terminus, but lacks 25 aa at the C terminus of the E protein.

Next, several deletion constructs were made in the N-terminal, C-terminal, or internal regions such that the translational reading frame was maintained (Fig. 1). The progressively shortened N-terminal deletions of the E protein were made by cleaving the *Pvu*II-*Eco*RI fragment with restriction enzymes *Tth*111I, *Sca*I, *Sph*I and *Nco*I (see Table I). These DNA fragments were cloned at the *Bam*HI site of pOTS vector to generate the clones, pTT15, pTT20, pTT25, and pTT30, respectively. These clones had the correct orientation for expression of the E polypeptides with progressively longer N-terminal deletions (see Table I). The internal deletion of the E protein was made by removing the region encoding 166 aa flanked by the two *Bam*HI restriction sites from the plasmid pTT15 (Fig. 1 and Table I). This plasmid encodes the polypeptide lacking aa 255 to 422 in the vicinity of the C terminus. The parent and the truncated expression constructs of E polypeptide were analyzed for their levels of expression in *E. coli* N5151 strain, and tested for their reactivities with the polyclonal HMAF (Fig. 2, panel A), as well as the 3H5 mAb (Fig. 2, panel B), by Western blotting. The expression plasmid pTT10 gave rise to an E polypeptide which was lower in size than the expected 51 kDa (Fig. 2A and 2B, lane 1), most likely due to its instability in *E. coli*. However, the polypeptide expressed from pTT15 was of expected size (see Fig. 2A and 2B, lane 2), suggesting that removal of the N-terminal 29 aa, including the seven hydrophobic residues, stabilized this protein considerably. The level of this polypeptide was high enough to be visualized directly by Coomassie blue staining of the polyacrylamide gel (data not shown). The E polypeptides containing progressively shorter N-terminal sequences expressed from the plasmids pTT20, pTT25 and pTT30, respectively, were loaded in lanes 3, 4 and 5, and the authentic E protein present in the DEN-2-infected mosquito (C6/36) cell lysates was loaded in lane C of Fig. 2. The levels of expression, and their immunoreactivities with HMAF and the 3H5 mAb of these E polypeptides varied. However, the size of the largest polypeptide expressed from a deletion construct, which was reactive with HMAF and/or the 3H5 mAb correlated well with its predicted value. The 3H5 mAb reacted with all the E polypeptides with N-terminal deletions, except with the one lacking aa 255 to 422 (Fig. 2B, lane 6). These data indicated that the anti-

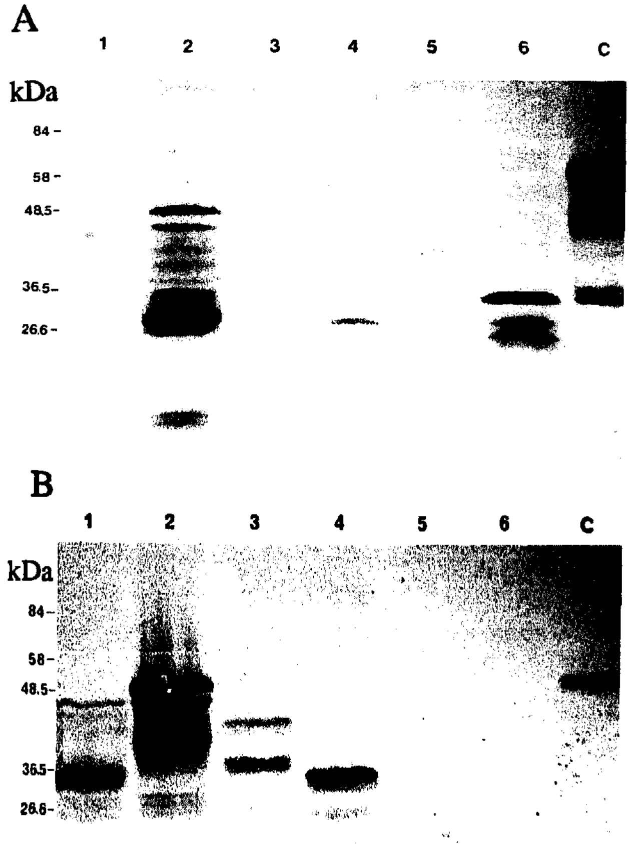

Fig. 2. Reactivities of E polypeptides with the polyclonal HMAF and DEN-2 type-specific 3H5 mAb. *E. coli* N5151 cells were transformed by the expression plasmids and tested for the synthesis of E polypeptides as described for pKC 30 and its derivatives (Rosenberg et al., 1983). Briefly, the bacterial cells were grown at 32°C to A_{650} = 1.0 in LB medium containing 100 μg Ap/ml, and then the temperature was shifted to 42°C by adding equal volume of LB medium prewarmed to 65°C. The culture was incubated at 42°C for an additional 60 min. The cells were harvested by centrifugation and washed once with TES buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.1 M NaCl). Total bacterial pellets resuspended in 300 μl of Laemmli (1970) sample buffer were heated to 100°C for 5 min and were analysed by 0.1% SDS/10% PAGE (Laemmli, 1970). Proteins were then transferred to nitrocellulose (Towbin and Gordon, 1984) in a buffer containing 25 mM Tris·HCl/150 mM glycine, pH 8.3/20% methanol/1% SDS at 200 mA for 4 h. Blots were blocked for 30 min in 22% nonfat dry milk/0.9% NaCl/10 mM Tris·HCl, pH 7.5. After washing with 50 mM Tris·HCl, pH 7.5/200 mM NaCl (TBS), the blots were then incubated sequentially with 1:500 dilution of HMAF (panel A), or 1:500 dilution of 3H5 mAb (panel B), goat anti-mouse IgG conjugated with alkaline phosphatase (Hyclone, Logan, UT), and a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Blots were washed 3 times between incubations with TBS for 10 min each. When color had developed sufficiently, the reaction was stopped by adding stop buffer (20 mM Tris·HCl/5 mM EDTA). Panels A and B, lanes 1–6: bacterial extracts containing E polypeptides expressed from pTT10, pTT15, pTT20, pTT25, pTT30 and pTT35, respectively. The E coding sequences contained in these plasmids are described under Fig. 1 and Table I. In lane C, the cell lysate from C6/36 (mosquito) cells infected with DEN-2 was loaded as a positive control. The preparation and characterization of 3H5 mAb and the polyclonal HMAF antibodies against DEN-2 viral antigens have previously been reported (Brandt et al., 1967; Henchal et al., 1982).

genic determinant of the protein recognized by the 3H5 mAb is located within a region of 166 aa (between aa 255 and 422) (Fig. 3).

(b) Fine mapping of the 3H5-binding site on the E protein by creating targeted deletions

In order to map the region recognized by the 3H5 mAb more precisely, we developed a strategy to construct a series of targeted deletions within this region of 166 aa using PCR (Saiki et al., 1988). It is a generally accepted notion that most, if not all, of the antigenic determinants reactive with antibodies are exposed to the surface of the antigen molecule (Todd et al., 1982; Sutcliffe et al., 1983; Benjamin et al., 1984; Berzofsky, 1985), and hence are hydrophilic. Based on this notion, algorithms for hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Parker et al., 1986) have been used to predict antigenicity. The hydropathy plot of the region of 166 aa containing the 3H5 mAb-binding site is shown in Fig. 3B. The predicted surface residues containing stretches of hydrophilic aa are indicated with negative values (Kyte and Doolittle, 1982). The hydropathy profile predicts three major hydrophilic regions (ten or more aa in length) at aa 285–298, aa 358–374 and aa 386–397, and three minor regions (five or more aa in length) at aa 325–333, aa 341–347 and aa 404–410. A set of oligos, 1–6, were synthesized corresponding to the regions shown in Fig. 3A, and used for PCR. The amplification reactions were carried out using the oligo 1, and any

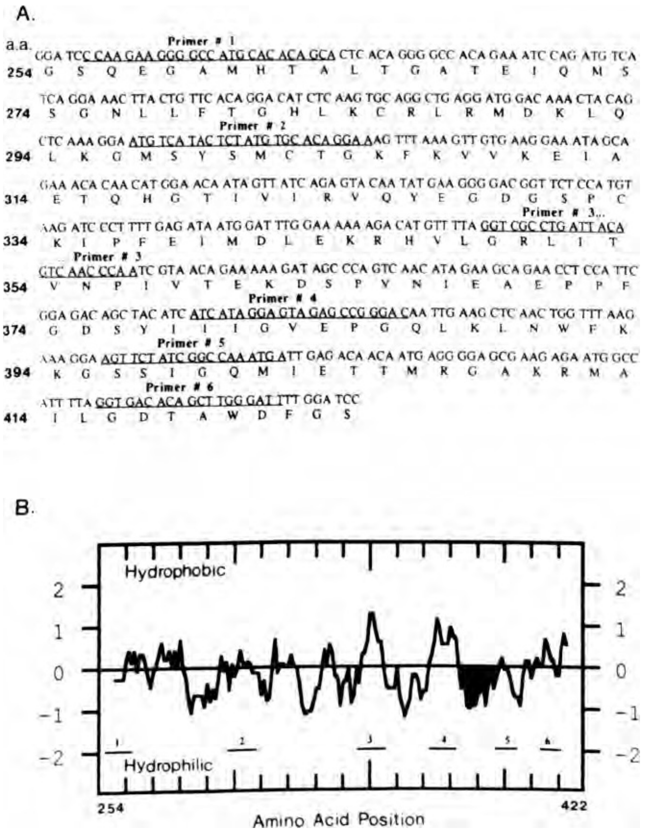

Fig. 3. Primary sequence and hydropathicity plot of a portion of DEN-2 E protein required for binding by 3H5 mAb. (A) The nt sequence of *Bam*HI fragment (nt 1697–2203 of DEN-2 genome encoding aa 254–422 of E protein; Irie et al., 1989) required for binding by 3H5 mAb is shown. The deduced aa sequence is given below the nt sequence. Regions chosen for the synthesis of primers, No. 1 to No. 6, for PCR are underlined. The sequences of primers No. 2 to No. 6 are complementary to those underlined. (B) Hydropathicity plot (Kyte and Doolittle, 1982) of the region shown in A. The numbered horizontal bars representing the PCR primers (1–6) correspond to the hydrophobic aa residues flanking the various hydrophilic (surface) regions, and their sequences are underlined in panel A. The filled-in hydrophilic region represents the region required for binding by 3H5 mAb, which was identified by fine mapping (see Fig. 4).

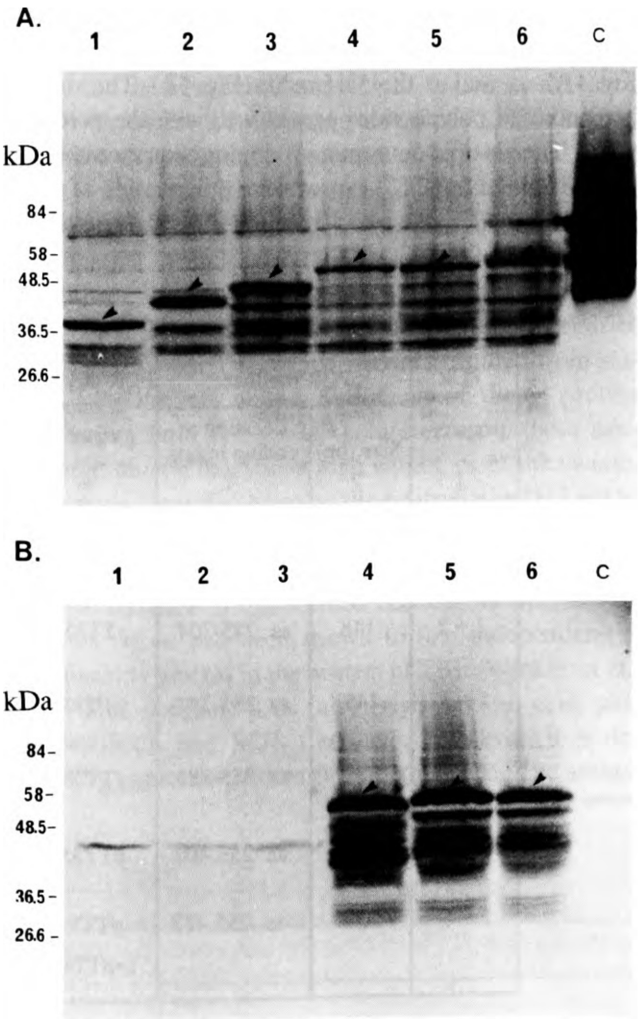

Fig. 4. Immunoreactivity of truncated E proteins. Total lysates from *E. coli* transformed by the various expression plasmids were electrophoresed in 0.1% SDS/10% PAGE system (Laemmli, 1970) and transferred to nitrocellulose paper. The filter was either reacted with the polyclonal HMAF (Fig. 4A), or with the 3H5 mAb (Fig. 4B). Lanes: 1, pTT35.2; 2, pTT35.3; 3, pTT35.4; 4, pTT35.5; 5, pTT35.6; 6, pTT15; C, DEN-2-infected mosquito (C6/36) cell lysate. The arrowheads indicate the largest truncated E polypeptides expressed from each of the plasmids.

one of the oligos 2–6 as primers, and the 506-bp region between the two *Bam*HI sites as the template. When the primer pairs of 1 and 2, 1 and 3, 1 and 4, 1 and 5, and 1 and 6 were used in PCR, DNA fragments of sizes 148 bp, 245 bp, 388 bp, 439 bp and 500 bp, respectively, were generated. Each amplified DNA fragment was ligated to the blunt-ended *Bam*HI sites of the expression plasmid pTT15 (see Table I). The plasmid clones with the correct orientations were selected by restriction enzyme digestions, and the accuracy of PCR-amplifications was verified by DNA sequence analysis. The plasmids selected for expression of E polypeptides with their aa coordinates are shown in Table II, and the *E. coli* N5151 strain transformed by these plasmids was analyzed.

The lysates of transformants obtained from deletion constructs were tested by Western blot for the reactivities of the truncated E polypeptides with polyclonal HMAF (Fig. 4A), as well as the 3H5 mAb (Fig. 4B). The sizes of the expressed polypeptides agreed well with the predicted values. The plasmid containing E-coding sequence between *Tth*111I-*Eco*RI (pTT15) expressed a polypeptide of about 48 kDa in size (Fig. 4A and 4B, lane 6). The plasmid pTT35 with the deletion of coding sequence between the two *Bam*HI sites expressed a polypeptide of about 36 kDa (Fig. 2A, lane 6). All the other clones obtained from in-frame insertion of PCR-amplified DNA fragments at the *Bam*HI site of the plasmid pTT35 expressed polypeptides in the size range of 36–48 kDa (Fig. 4A, lanes 1–6; see also Table II). When these E polypeptides were tested for their reactivities with the 3H5 mAb, only the 47- and 48-kDa polypeptides were immunoreactive (see Fig. 4B, lanes 4 and 5, and Table II). The E polypeptide having a deletion between aa 385–423 did not react with the 3H5 (Fig. 4B, lane 3), but the one having the deletion between aa 401–423 did react (Fig. 4B, lane 4). Therefore, the 3H5 binding site is located between aa 385–401. In Fig. 4B, lane 5, the E polypeptide expressed from the plasmid pTT35.6, which was the equivalent of pTT15 in lane 6, was loaded. This polypeptide also reacted with the 3H5 mAb as expected. These results suggested that the hydrophilic region containing residues QLKLNWFKKGSS (aa 386–397) between the two PCR primers No. 4 and No. 5 (see Fig. 3B, and lane 4 in Fig. 4B), were required for the immunoreactivity with the 3H5 mAb.

TABLE II

Map positions of E polypeptides encoded by expression clones

PCR primers [a]	PCR product: Size (bp) [b]	PCR product: Location of coding region	PCR-targeted deletion clone [c]	aa coordinates of E polypeptides [d]	3H5 [e] reactivity	Size of E polypeptide kDa
1	2	3	4	5	6	7
-	-	-	pTT35	22 254 423 470; B B	-	36
1+2	148	aa 255-304	pTT35.2	22 304 423 470	-	38
1+3	245	aa 255-357	pTT35.3	22 357 423 470	-	41
1+4	388	aa 255-385	pTT35.4	22 385 423 470	-	45
1+5	439	aa 255-401	pTT35.5	22 401 423 470	+	47
1+6	500	aa 255-423	pTT35.6 (=pTT15)	22 423 470	+	48

[a] The sequences of PCR primers No. 1–6 are given in Fig. 3A (underlined). The *Bam*HI fragment (nt 1697–2203) was used as template (100 ng) in a reaction mixture (100 μl) that contained dNTP (125 μM), one μM each of the two primers, and 4 units of *Taq* polymerase. PCR was carried out for 25 cycles.

[b] Sizes of the amplified DNA from each of the PCR between the primer pair 1:2, 1:3, 1:4, 1:5 and 1:6, respectively. They were ligated to the expression plasmid, pTT15 (Table I), which was previously digested with *Bam*HI and blunt-ended by treatment with PolIk enzyme.

[c] Screening of the transformants for the proper orientation of the insert was carried out by restriction analysis, and the PCR-amplified regions (shown as thick lines in column 5) in each of the clones were verified by nt sequence analysis.

[d] The numbers refer to aa residues in the E protein. B denotes the B domain of the E protein. The dashed lines indicate the regions deleted in the E protein, and the thick lines indicate the region encoded by the PCR-amplified DNA.

[e] The (+) and (−) signs indicate the presence or absence of reactivity with the 3H5 mAb.

(c) Characteristics of the region of DEN-2 E required for binding by the 3H5 mAb

It has been reported that many of the mAbs to 17D strain of YF were able to distinguish between the wt and the vaccine strains of the virus by neutralization and/or HI assays. There were also differences between individual 17D vaccine strains (Buckley and Gould, 1985; Schlesinger et al., 1983). However, the 3H5 mAb reacted with several DEN-2 strains from different geographical areas, indicating that this antibody is not DEN-2 strain-specific (Gentry et al., 1982). It is interesting to note that the aa sequences among the three DEN-2 strains: NGS-C (Irie et al., 1989), Jamaica strain (Deubel et al., 1986), and PR-159/S1 (Hahn et al., 1988) in the region required for the 3H5-binding are identical except for the substitution of the D residue in PR-159/S1 for N (aa 390) in the other two strains. It was found that the 3H5 was just as effective in neutralizing either of the two DEN-2 strains, NGS-C, and PR-159/S1 (R. Putnak, unpublished results), suggesting that N/D substitution in PR-159/S1 did not contribute to any significant change in the immunoreactivity of the 3H5 mAb. However, earlier studies have shown that the 3H5 was not effective in neutralizing DEN-1 (Henchal et al., 1985). When the sequence of the 3H5-binding region of DEN-2 was compared with the corresponding regions of DEN-1 (Mason et al., 1987b), DEN-3 (Osatomi and Sumiyoshi, 1990), and DEN-4 (Zhao et al., 1986), the variations in the sequence are the greatest in DEN-4 and DEN-3 with 4 aa changes, whereas in DEN-1, there are only two changes (see Table III). Since the DEN-2 E sequence corresponding to this 3H5-binding site differs from that of the DEN-1 E sequence only in the N-terminal half of the region, this portion of the sequence may be critical for the 3H5 recognition. Roehrig et al. (1990) reported that the peptide from aa 388–400 neither bound to the 3H5, nor elicited neutralizing antibody in mice. It should be noted that the peptide 388 differs from the 3H5 peptide studied here in lacking two N-terminal aa, but having three additional C-terminal residues. It is possible that the peptide 388 may assume a different conformation from that of the 3H5 peptide (aa 386–397) (see also section **e**). Another important difference is that Roehrig et al. (1990) used mice for immunization with the synthetic peptide, whereas rabbits were used in this study.

(d) The region of the 3H5-binding site of the DEN-2 E protein and the neutralization escape mutants of the E protein of TBE virus map in the B domain

Structural analysis of the E protein of WN (Novak and Wengler, 1987), and TBE (Heinz et al., 1983a,b; Mandl et al., 1989) revealed three distinct antigenic domains, A, B and C. In TBE, it has been shown that the domain A essentially contains flavivirus-cross reactive epitopes, as well as some subtype-specific epitopes, which are also present in domain C. Epitopes of domain B, on the other hand, are specific for the tick-borne complex of flaviviruses (Heinz, 1986), which were lost upon reduction of disulfide bonds, suggesting that the three-dimensional structure of the E protein is important for the integrity of these determinants. In order to map the antigenic determinant(s) involved in neutralization, Mandl et al. (1989) isolated and sequenced the regions encoding E and M proteins of the escape variants of TBE virus, which were selected in the presence of neutralizing mAbs. Two of the variants resulted from single aa substitutions in the B domain of the E protein ($Ser^{389} \rightarrow Arg$ and $Tyr^{384} \rightarrow His$). Interestingly, these neutralization escape mutations map within, or in the vicinity of the region identified by us as the neutralizing 3H5 mAb-binding site.

The domain B of DEN-2 E protein (aa 301–395) contains one disulfide bridge formed between aa 302 and aa 333. This region has been shown to fold independent of other disulfide bridges in the protein of TBE (Winkler et al., 1987). The domain B is also resistant to acid pH, guanidine-HCl, and SDS treatment. However, it is destroyed by reduction and carboxymethylation. The antige-

TABLE III

Comparison of aa sequences among DEN serotypes in the region of 3H5-binding site

DEN peptide[a]	Sequence[a]														
DEN-2 (JAM)			^{388}K	L	N	W	F	K	K	G	S	S	I	G	Q^{400}
DEN-2 (NGS-C)	^{386}Q	L	K	L	N	W	F	K	K	G	S	S^{397}			
DEN-2 (PR159-S1)	–	–	–	–	D	–	–	–	–	–	–	–			
DEN-1	A	–	–	–	S	–	–	–	–	–	–	–			
DEN-4	A	–	T	–	H	–	–	R	–	–	–	–			
DEN-3	A	–	–	I	–	–	Y	R	–	–	–	–			

[a] The sequences of the DEN-E in the regions corresponding to the 3H5-binding site are shown. The sequences of the 15 aa region between aa 386–400 are identical between DEN-2 (JAM) (Deubel et al., 1986), and DEN-2 (NGS-C) (Irie et al., 1989). The synthetic peptide, aa 388–400 was used by Roehrig et al. (1990) (see section c). The other sequences are derived from: DEN-2 (PR159-S1; Hahn et al., 1988); DEN-1 (Mason et al., 1987b); DEN-4 (Zhao et al., 1986); DEN-3 (Osatomi and Sumiyoshi, 1990). The dashes denote identical aa in that position.

nicity of the domain B could be restored by the removal of the reducing agent (Heinz, 1986), suggesting that this domain has the potential to renature very rapidly. This property of the domain B is consistent with our observation that the 3H5 mAb reacted with the E polypeptides subsequent to boiling in the presence of 2-mercaptoethanol. However, the reactivity of the 3H5 mAb reduced upon carboxymethylation (data not shown). This result suggests that the conformation of the B domain may play a role in presenting this linear sequence to the surface of the protein molecule for efficient binding by the 3H5 mAb.

(e) Synthetic peptide binds to the 3H5 mAb with specificity

Since the 3H5 is a DEN-2 type-specific anti-E mAb having a high neutralizing titer (Henchal et al., 1985), we were interested to examine whether the linear sequence of the E protein recognized by the 3H5 mAb can elicit neutralizing immune response. We synthesized a peptide of 13 aa residues (including the N-terminal Cys added for coupling to KLH) corresponding to the 3H5-binding site, and used it to immunize rabbits. The reactivity of the rabbit antisera raised against the peptide was monitored by

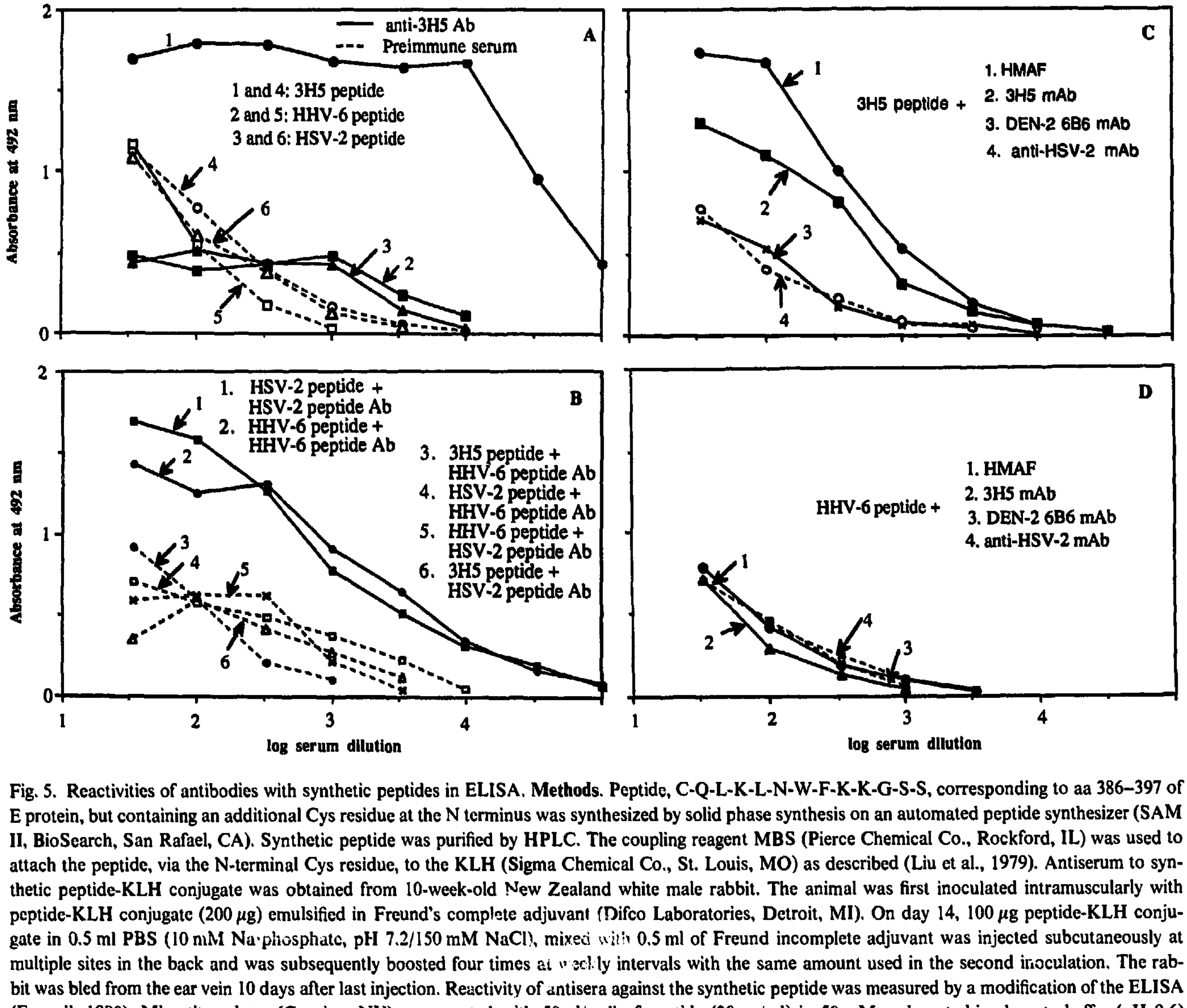

Fig. 5. Reactivities of antibodies with synthetic peptides in ELISA. **Methods.** Peptide, C-Q-L-K-L-N-W-F-K-K-G-S-S, corresponding to aa 386–397 of E protein, but containing an additional Cys residue at the N terminus was synthesized by solid phase synthesis on an automated peptide synthesizer (SAM II, BioSearch, San Rafael, CA). Synthetic peptide was purified by HPLC. The coupling reagent MBS (Pierce Chemical Co., Rockford, IL) was used to attach the peptide, via the N-terminal Cys residue, to the KLH (Sigma Chemical Co., St. Louis, MO) as described (Liu et al., 1979). Antiserum to synthetic peptide-KLH conjugate was obtained from 10-week-old New Zealand white male rabbit. The animal was first inoculated intramuscularly with peptide-KLH conjugate (200 μg) emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). On day 14, 100 μg peptide-KLH conjugate in 0.5 ml PBS (10 mM Na·phosphate, pH 7.2/150 mM NaCl), mixed with 0.5 ml of Freund incomplete adjuvant was injected subcutaneously at multiple sites in the back and was subsequently boosted four times at weekly intervals with the same amount used in the second inoculation. The rabbit was bled from the ear vein 10 days after last injection. Reactivity of antisera against the synthetic peptide was measured by a modification of the ELISA (Engvall, 1980). Microtiter plates (Corning, NY) were coated with 50 μl/well of peptide (20 μg/ml) in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 37°C. The antigen-coated wells were then washed 3 times with 0.85% NaCl/0.05% Tween 20. After washing, the plates were blocked with 5% BSA in PBS/Tween 20 (100 μl/well for 1 h at 37°C. Plates were then incubated sequentially with 50 μl/well of diluted antiserum in PBS/Tween 20/1% BSA, goat anti-rabbit Ab conjugated to horseradish peroxidase (Hyclone, Logan, UT), and the substrate orthophenylenediamine (Sigma Chemical Co., St. Louis, MO). Plates were washed between incubations with 0.05% Tween 20/0.85% NaCl. The final enzyme reaction was stopped with 2N H_2SO_4, and the color change was measured at 492 nm on a Titertek Multiskan plate reader (Flow Laboratories, McLean, VA). In ELISA, *A* at 492 nm is plotted against the log serum dilution.

ELISA. The anti-3H5 peptide antibody reacted specifically with the 3H5 peptide at the dilution of 1:10000, and no reactivity was seen with preimmune sera (Fig. 5A, profiles 1 and 4). As a control, we tested the reactivities of two other peptides from the glycoprotein sequences of HHV-6 and HSV-2gG with the anti-3H5 peptide antibody (Fig. 5A, profiles 2 and 3, respectively). The reactivities of these three peptides with the preimmune sera are shown by profiles 4–6 in Fig. 5A. The reactivities of the anti-HSV-2gG peptide antiserum, as well as the anti-HHV-6 peptide antiserum were lower, compared to that of the anti-3H5 peptide serum tested with the respective homologous peptides (Fig. 5B, profiles 1 and 2 vs. profile 1 in Fig. 5A). The reactivities of the heterologous peptides with the peptide antisera (e.g., the anti-3H5 peptide antibody with the HHV-6 peptide, and the anti-HHV-6 antibody with the 3H5 peptide etc.), were all much lower and grouped together (see 1:1000 dilution, profiles 2 and 3 in Fig. 5A and profiles 3 to 6 in Fig. 5B). Next, the reactivities of the 3H5 peptide with the various antibodies were compared. As shown in Fig. 5A, the reactivity between the 3H5 peptide and the anti-3H5 peptide serum is the greatest, whereas its reactivity with the HMAF and the 3H5 antibody were threefold and fivefold less, respectively (Fig. 5C, profiles 1 and 2). However, when the ELISA values at 1:330 were compared for the reactivities of (i) the 3H5 peptide vs. the 3H5 mAb (profile 2), (ii) the 3H5 peptide vs. another DEN-2 E-specific 6B6 mAb (profile 3), or (iii) the 3H5 peptide vs. the nonspecific mAb (13αC5) raised against the HSV-2gG protein (profile 4), the reactivity of (i) was about fivefold higher than Nos. 2 and 3 (see Fig. 5C). The reactivities of an unrelated HHV-6 peptide with the HMAF, the 3H5 mAb, the DEN-2 specific 6B6 mAb or the HSV-2gG-specific 13αC5 mAb, were all very low and similar to those of the 3H5 peptide vs. the DEN-2-6B6 mAb and the 13αC5 mAb (Fig. 5D, profiles 1–4 vs. Fig. 5C, profiles 3 and 4). In addition, the specificity of this immunoreactivity of the 3H5-peptide was also tested by using other unrelated mAbs as controls: a mAb against DEN-2 NS-1 protein (3E9) and the A16 mAb against HSV-2 glycoprotein D (Balachandran and Hutt-Fletcher, 1985) and the data indicated that the reactivity between the 3H5 peptide and the 3H5 mAb is very specific (data not shown). These data clearly supported that the 12-aa region (aa 386–397) of the DEN-2 E protein constituted at least a portion of the antigenic determinant recognized by the 3H5 mAb.

(f) Anti-3H5 peptide antibody reacts with the authentic DEN-2 E protein

Next, we examined whether the rabbit anti-3H5 peptide antiserum could recognize the authentic E glycoprotein from the DEN-2-infected cells. Monkey kidney (CV-1) cells were infected with DEN-2, and the cells were metabolically

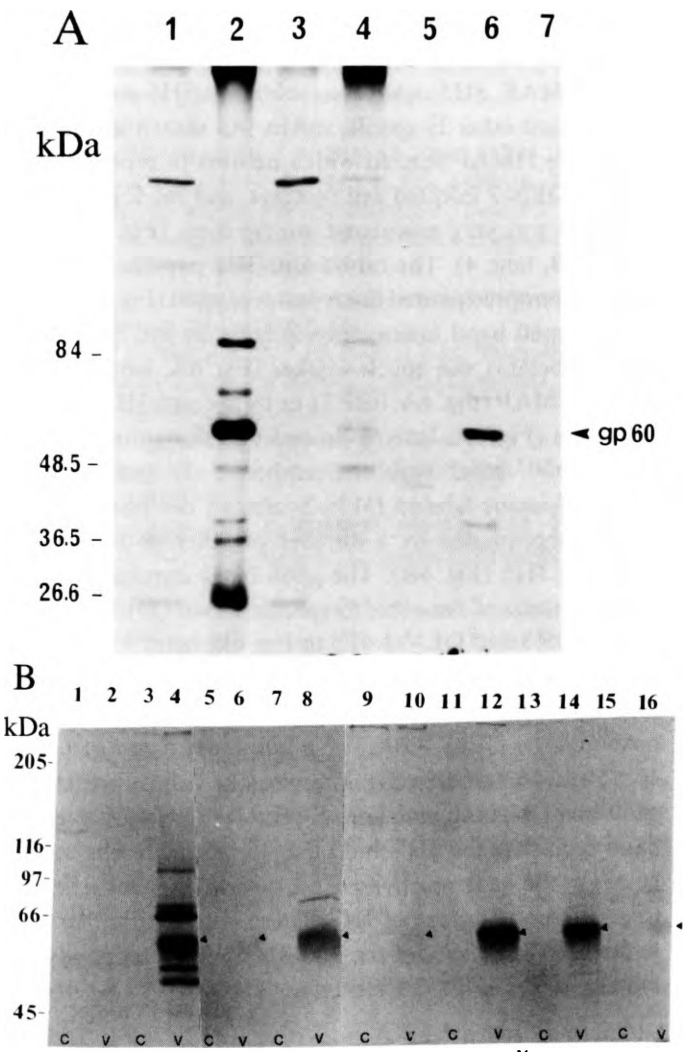

Fig. 6. Immunoprecipitation of the E glycoprotein from [^{35}S]methionine-labeled DEN-2-infected cell lysate. CV-1 cells were grown at 37°C in DMEM containing 10% fetal bovine serum. The monolayer of cells in 75 cm^2 flasks were infected with DEN-2 (NGS-C) at m.o.i. of approx. 1 pfu/cell. After 72 h post-infection, [^{35}S]methionine (10 μCi/ml) was added in methionine-deficient medium for 3 h. The cells were collected by scraping and low speed centrifugation. Cells were solubilized in RIPA buffer (50 mM Tris·HCl pH 7.5/150 mM NaCl/1% DOC/0.5% NP40/ 0.1 mM PMSF), and sonicated for 1 min. The lysates were centrifuged at 100000 × g for 1 h. Supernatants were mixed with appropriate antibodies and protein A-agarose beads. The precipitates were washed, dissociated by boiling in sample buffer, and analysed by 0.1% SDS/10% PAGE (Laemmli, 1970). Gels were fixed, stained and processed for fluorography. (Panel **A**) Soluble cell lysates from uninfected CV-1 cells (lanes **1**, **3** and **5**), and DEN-2-infected cells (lanes **2**, **4**, **6** and **7**) were reacted with the following antibodies: HMAF (lanes **1** and **2**); 3H5 mAb (lanes **3** and **4**); rabbit antiserum to the synthetic peptides (lanes **5** and **6**); rabbit preimmune serum (lane **7**). The mobility of molecular weight markers is indicated. (Panel **B**) Labeled cell lysates as in panel A, but using additional E-specific mAbs were analyzed by immunoprecipitation. **C** and **V** denote the control uninfected and DEN-2-infected CV-1 cell lysates, respectively. Lanes: **1** and **2**, mouse preimmune serum; **3** and **4**, HMAF; **5** and **6**, 3H5 mAb; **7** and **8**, E-specific mAb, DEN-2-6B6; **9** and **10**, E-specific mAb DEN-1-13B7; **11** and **12**, E-specific mAb, DEN-2-2H3; **13** and **14**, E-specific mAb DEN-2-4E5; **15** and **16**, E-specific mAb DEN-1-9D12 (Gentry et al., 1982; Henchal et al., 1982). The arrowheads in panels **A** and **B** denote the E protein (gp60).

labeled with [^{35}S]methionine. Infected and uninfected cell lysates were used for immunoprecipitation using the polyclonal HMAF, 3H5 mAb, the rabbit anti-3H5 peptide antiserum, and other E-specific mAbs. As shown in Fig. 6A and B, the HMAF reacted with a number of proteins specific for DEN-2 infected cell proteins, and the E glycoprotein gp60 was very prominent among them (Fig. 6A, lane 2, and 6B, lane 4). The rabbit anti-3H5 peptide antibody also immunoprecipitated the prominent gp60 (Fig. 6A, lane 6). The gp60 band immunoprecipitated by the 3H5 mAb (IgG_1 subclass) was much weaker (Fig. 6A, lane 4) than that by HMAF (Fig. 6A, lane 2), or by the anti-3H5 peptide antiserum (Fig. 6A, lane 6). In order to show that the putative gp60 band was the authentic E protein, the [^{35}S]methionine-labeled DEN-2-infected cell lysates were immunoprecipitated by a number of other anti-E mAbs including 3H5 (Fig. 6B). The gp60 band immunoprecipitated by some of the other E-specific mAbs (DEN-2-6B6, DEN-2-2H3 and DEN-2-4E5 in Fig. 6B, lanes 7 and 8, 11 and 12, and 13 and 14, respectively) was much stronger than that obtained with the 3H5 mAb, the DEN-1-13B7 mAb and the DEN-1-9D12 (Fig. 6B, lanes 5 and 6, 9 and 10, 15 and 16, respectively). These results indicated that the gp60 band was the authentic E protein. The weaker gp60 band seen with the 3H5 and other E-specific mAbs might be due to the weak reactivity of the protein A-agarose beads to a different subclass of IgG. These data clearly demonstrated the specificity of the anti-3H5 peptide antibody in binding to the DEN-2 E protein.

(g) Anti-3H5 peptide antibody has neutralizing activity

Rabbit anti-serum against the 3H5 peptide was next tested for its ability to neutralize DEN-2 in a plaque reduction neutralization test (PRNT) in a complement-independent reaction as described by Russell and Nisaluk (1967). The anti-peptide antibodies showed neutralization of the infectivity of DEN-2 virus (Table IV) with the 70% neutralizing antibody titer ($PRNT_{70}$) of 1:80 reproducibly. With the sera from a different rabbit, a $PRNT_{70}$ neutralization titer of only 1:23 was obtained in duplicate experiments (data not shown). These results strongly supported that the 3H5-binding site on the E protein represents a portion of the antigenic determinant of the E protein involved in neutralization.

Previous studies using synthetic peptides to elicit antisera reactive with the hepatitis B surface antigen have shown that the determinant carried within a short peptide is sufficient to elicit reactivity against a much larger protein molecule with a complex tertiary and quaternary structure (Sutcliffe et al., 1983). Mason et al. (1987a; 1989) expressed the cDNA encoding E protein of JE in *E. coli* using λgt11 system to localize the antigenic determinants recognized by mAbs. Using BAL31 for deletion mutagenesis from the 5′ and 3′ ends of the cDNA encoding the E protein, a 95-aa fragment between Met^{303}-Lys^{398} was found to be the minimal structure capable of binding to any of ten mAbs, and the immunoreactivity of this fragment was dependent on the formation of disulfide bonds. However, a recombinant fusion protein containing this 95-aa sequence reacted with the neutralizing and protective mAb in direct binding assays, but it failed to induce a neutralizing immune response when injected into mice (Mason et al., 1989). Interestingly, this antigenic domain mapped in JE-E protein falls within the corresponding domain of DEN-2 E reactive with the 3H5 mAb. However, the ability of our synthetic peptide to induce neutralizing antibody in rabbits might be due to conformational differences assumed by the short synthetic peptide, compared to the antigenic domain of 95-aa expressed in *E. coli* in the form of a fusion protein (Mason et al., 1989). Similar differences have been observed by others. For example, Eisenberg et al. (1985) identified an antigenic site in the aa 268–287 sequence of glycoprotein

TABLE IV

Plaque reduction neutralization test of rabbit anti-peptide serum[a]

Antibody dilution[b]:	1:10	1:20	1:40	1:80	1:160	1:320	1:640
	Percent of plaque reduction[c]						
Experiment 1	91.0	79.6	70.5	71.2	61.5	51.3	28.2
Experiment 2	87.7	78.1	75.3	76.9	63.0	52.1	35.6
Experiment 3	95.8	93.0	88.3	80.3	69.0	52.6	27.4
Experiment 4	96.2	93.5	86.9	73.8	59.6	46.15	23.1

[a] Rabbit anti-peptide serum obtained from rabbit immunized with 3H5-reactive peptide.

[b] Serum dilution made in Hanks' balanced salt solution containing 2% fetal bovine serum.

[c] Mean percent plaque reduction calculated from the average of duplicate titration as $\left[1 - \frac{\text{plaques with immune serum}}{\text{plaques with nonimmune serum}}\right] \times 100$ (i.e., expressed as percent).

D of HSV-1, by showing reactivity of this peptide with the mAb against glycoprotein D. This peptide was conjugated to KLH via C-terminal Cys, but failed to elicit antibody in rabbits that could recognize either the glycoprotein D or the intact virions. In contrast, Strynadka et al. (1988) found that antibodies to the KLH, conjugated via the N-terminus of the peptide encoding aa 267–276, recognized both glycoprotein D and HSV-1 virions. They also neutralized the infectivity of HSV-1. To explain the differences between these results obtained with two closely related peptides from the same region, Strynadka et al. (1988) speculated that the longer peptide (267–287 aa) might assume a conformation distinct from that of the native epitope.

(h) Conclusions

The method described in this report is generally applicable to define precisely any short linear sequence of a protein molecule, which is required for the presentation of a larger antigenic determinant recognized by a mAb. The notable unique feature of this method is that the truncated polypeptides are not fused to the *E. coli* β-galactosidase, and therefore the antigenic determinants of interest are not subjected to any major conformational changes during folding of the proteins. An alternative method for mapping the neutralizing determinants involves the isolation of a large number of neutralization escape variants, their propagation, cDNA cloning and sequence analysis of these variants in order to be able to define the antibody binding site. In some cases, selective synthetic peptides based on such data have failed to elicit a neutralizing antibody response in an appropriate host due to the possibility that in the immune-selected variant, a conformational change might alter a neutralization epitope at a distant site (Wilson et al., 1990).

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