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Processing of dengue virus type 2 structural proteins containing deletions in hydrophobic domains

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Summary. The 5′ end of the genome of the dengue virus type 2 encoding the structural proteins was expressed using recombinant vaccinia virus. Three additional recombinants derived by deletion of selected dengue sequences within the parental construct were also expressed. They were designed to assess the role of hydrophobic domains in the processing of the viral polyprotein in intact cells. The first construct contained a deletion of nucleotides encoding most of the C protein; nucleotides encoding the hydrophobic domain at the carboxy terminus were retained. The second and third constructs contained smaller deletions of 72 bp and 129 bp encoding hydrophobic domains at the carboxy termini of C and prM respectively. Indirect immunofluorescence and radioimmunoprecipitation were used to detect prM and E in cells infected with recombinant viruses. The results showed that deletion of 90% of C had no apparent effect on the processing of prM and E, and that the signal sequence for E at the carboxy terminus of prM was active in the absence of the upstream signal sequence for prM at the carboxy terminus of C. Deletion of the hydrophobic sequences preceding the amino terminus of E prevented cleavage at the prM-E junction. These results obtained using infected cells were consistent with the published findings for the translation of flavivirus RNA in vitro, and indicated the importance of membrane association in the cleavage of structural proteins from the flavivirus polyprotein. In addition, cells infected with the recombinant virus containing the large deletion in the C coding region released the E glycoprotein into the culture medium.

Introduction

Dengue type 2 virus (DEN-2) is a member of the dengue serocomplex. It belongs within the family *Flaviviridae*, which includes some 70 viruses [8, 37]. As for all flaviviruses, the DEN-2 virion is an enveloped spherical particle of 40–60 nm containing three proteins, namely the membrane-associated protein M of M_r

8,000, the envelope glycoprotein E of M_r 60,000, and an internal core protein C of M_r 13,000 [36]. A glycoprotein prM (M_r 20,000), which is cleaved late in viral morphogenesis to generate M, is found in infected cells and also in slowly sedimenting haemagglutinating particles (SHA) [29].

The flavivirus genome consists of an infectious single stranded RNA molecule of nearly 11.0 kb; no subgenomic forms have been identified [4, 23]. Complete and partial nucleotide sequences have been published for a large number of flaviviruses. From these data, it is apparent that the genome contains a single open reading frame (ORF) encoding all the viral proteins in the order 5′ C-prM(M)-E-NS1-NS2A-NS2B-NS4A-NS4B-NS5 3′, and is translated as a single polyprotein [9, 14, 16, 25]. Individual proteins are cleaved from the nascent polyprotein by proteinases of either cellular or viral origin [9, 25].

Identification of the amino and carboxy termini of the viral proteins by direct amino acid analyses has confirmed the gene order [1, 2, 31, 32, 38], and indicated the nature of cleavage events involved in the release of viral structural polypeptides from the polyprotein [7, 9, 25, 35]. The amino terminus of prM follows a single hydrophobic stretch of amino acids which acts as a signal sequence for translocation of the polyprotein through the membrane of the rough endoplasmic reticulum (RER). Two hydrophobic domains are located at the carboxy terminus of prM, separated by a single charged residue. The first of these may function as an anchor or stop transfer sequence for prM, the second to signal the translocation of E into the RER membrane. The carboxy terminus of E also contains two such domains with analogous functions for E (anchor) and NS 1 (signal). The cleavage sites at the amino termini or prM, E, and NS 1 satisfy the "(− 3, − 1) rule" for the action of a cellular signal peptidase active in the lumen of the RER [34].

Evidence for the importance of signal sequences and signal peptidase in flavivirus protein processing was provided by experiments in which Kunjin virus-infected cells treated with hydroxyleucine produced novel high molecular weight polyproteins. Incorporation of this leucine analogue disrupted the hydrophobic nature of the signal sequences, thus preventing membrane insertion and aborting cleavage by signal peptidase [10]. Using recombinant vaccinia viruses expressing cDNA of dengue virus type 4, Falgout et al. [12] showed that the hydrophobic region at the carboxy terminus of E acted as a signal sequence for the adjacent nonstructural protein NS 1. When virion RNA or RNA transcripts of flavivirus cDNA were translated in vitro, cleavage of structural proteins occurred only in the presence of microsomal membranes [21, 27, 33]. This indicated a requirement for membrane translocation and the involvement of signal peptidases in protein processing. Regions encoding the viral nonstructural proteins were unnecessary for cleavage of structural proteins expressed either in vitro [21, 27], or by recombinant vaccinia viruses [6, 11, 15, 39].

In the work described below, the role of the hydrophobic domains of C and prM in the processing of DEN-2 structural proteins in intact cells was further

investigated. Specific questions addressed were: (*i*) whether any portion of the C protein apart from the hydrophobic carboxy terminus was required for the processing of prM and E, and (*ii*) whether signal sequences within the polyprotein were capable of independent function. Recombinant vaccinia viruses encoding the structural genes of DEN-2 and containing appropriate deletions were constructed. The products synthesised in infected cells were analysed by indirect immunofluorescence and radioimmunoprecipitation using monoclonal and polyclonal antibodies.

Materials and methods

Cell lines and viruses

L 929 mouse fibroblast cells were grown in medium M 199 with Hank's salts, Hepes buffer, 2.0 mM L-glutamine, and 5% foetal calf serum (FCS): Human thymidine kinase mutant (TK^-) 143 B cells [24] were grown and maintained in MEM with Earle's salts supplemented with 5% FCS. During selection for recombinant TK^- vaccinia viruses, 5'-bromodeoxyuridine (BUdR) was added to the medium at a final concentration of 25 μg/ml.

The WR vaccinia virus strain (American Type Culture Collection) was obtained from Dr. D. Boyle (Australian Animal Health Laboratories). The virus was prepared as a high titre stock in L 929 cells of approximately 10^8 plaque forming units (PFU) and stored in small aliquots at − 70 °C. Recombinant vaccinia viruses were selected and grown in TK^- 143 B cells. Prior to infecting cell monolayers, all vaccinia virus inocula were pretreated with trypsin for 30 min at 37 °C.

Dengue virus type 2 strain PUO-218 [13] was replicated in Vero cells. These cells were grown in MEM with 5% FCS as described above. After infection, the cells were maintained in the same medium, but with 0.1% BSA and further supplemented with 0.1 mM each of nonessential amino acids.

Cloning and preparation of DNA

Viral cDNA to be expressed in the vaccinia system was first inserted into the vaccinia virus transfer vector, pBCBO6*. The plasmids containing cDNA were designated pBG and are described below. The plasmic pBCBO6* was constructed by Boyle and coworkers from pUC 9 [5] and contains a fragment of vaccinia virus DNA encoding the viral thymidine kinase (TK) gene. Within this region is a multiple cloning site immediately downstream of the early/late P-7.5 vaccinia promoter.

Construction of recombinant plasmids

To obtain expression of all the DEN-2 structural gene products, cDNA fragments from two plasmids containing viral cDNA inserts in pUC 8 were ligated at a common *Pst* I site and inserted into the vaccinia transfer vector. However, additional *Pst*I sites in both clones necessitated intermediate cloning steps (details not shown). The regions encoded by the viral cDNA are shown in Fig. 1. The cDNA contained within pDG 1007 encoded the carboxy terminus of C through to the amino terminus of NS 1. The insert of pDW 69 contained 67 untranslated nucleotides immediately upstream of the start codon of the C protein, all of C and the first 18 amino acids of prM. The DEN-2 open reading frame was terminated by a stop codon in the pUC 8 sequence.

The two clones were from different cDNA libraries; pDW 69 was derived from the New Guinea C strain, and pDG 1007 from the PUO-218 strain of DEN-2. Together they covered the structural region. Since nucleotide sequencing of these strains indicated a high level of

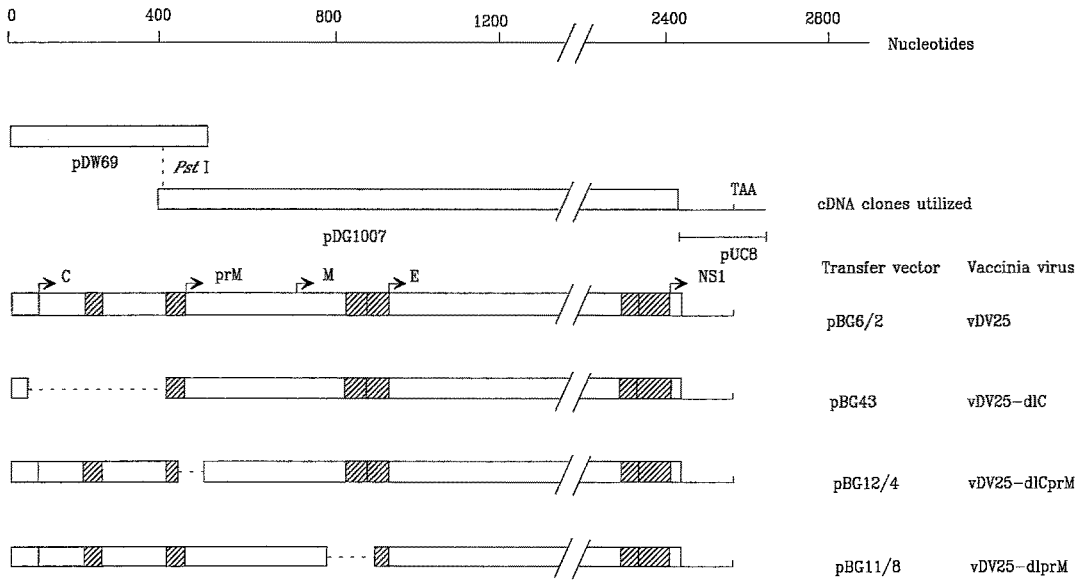

Fig. 1. DEN-2 cDNA constructs expressed using recombinant vaccinia viruses. Two cDNA fragments encoding the structural proteins were joined at a common *Pst*I site. Plasmid pUC 8 DNA containing a stop codon is shown as single line at the right hand end of pDG 1007. Deletions in the full length construct are indicated by single broken lines. Shaded boxes indicate sequences encoding the major hydrophobic regions of the proteins

conservation of the deduced amino acid sequence [13], it was anticipated that use of cDNA from these two strains would not adversely affect the processing of the encoded proteins.

The pBCBO6*-derived plasmid pBG 6/2 containing the complete DEN-2 structural protein coding region was modified to generate three additional deleted constructs pBG 43, pBG 12/4, and pBG 11/8. The positions of the deletions and the designations of the corresponding recombinant vaccinia viruses (vDV) are shown in Fig. 1. The sequence of each deletion and flanking nucleotides is shown in Fig. 2.

For pBG 43 a deletion was generated within the C protein coding region. The construct retained sequences encoding the hydrophobic domain at the carboxy terminus of C. Although the initiation codon for C was eliminated, three potential in-frame start codons were located near the beginning of the cDNA sequence (Fig. 2).

Plasmid pBG 12/4 contained a 72 base pair (bp) deletion between *Bcl*I and *Acc*I sites produced by several intermediate cloning steps. The deletion encoded the last two thirds of the hydrophobic domain at the carboxy terminus of C and the amino terminal 15 amino acids of prM (Fig. 2).

To generate pBG 11/8, a deletion between a *Bst*NI and a *Pvu*II site was created by intermediate cloning steps. The deletion encoded all of the first hydrophobic domain of prM and eight out of fifteen amino acids from the second. The cleavage site at the amino terminus of E was not deleted (Fig. 2).

In order to check the constructs, selected regions of the viral cDNA inserts in the pBG plasmids were sequenced by the dideoxy chain termination method [28]. The primers used had the following sequences:

5′ ^{522}TGCACATCTTCACGCCATCC^{541} 3′ and

5′ ^{976}CAGCTTCCTCCTGAAACCC^{995} 3′

(numbering according to Irie et al. [16]).

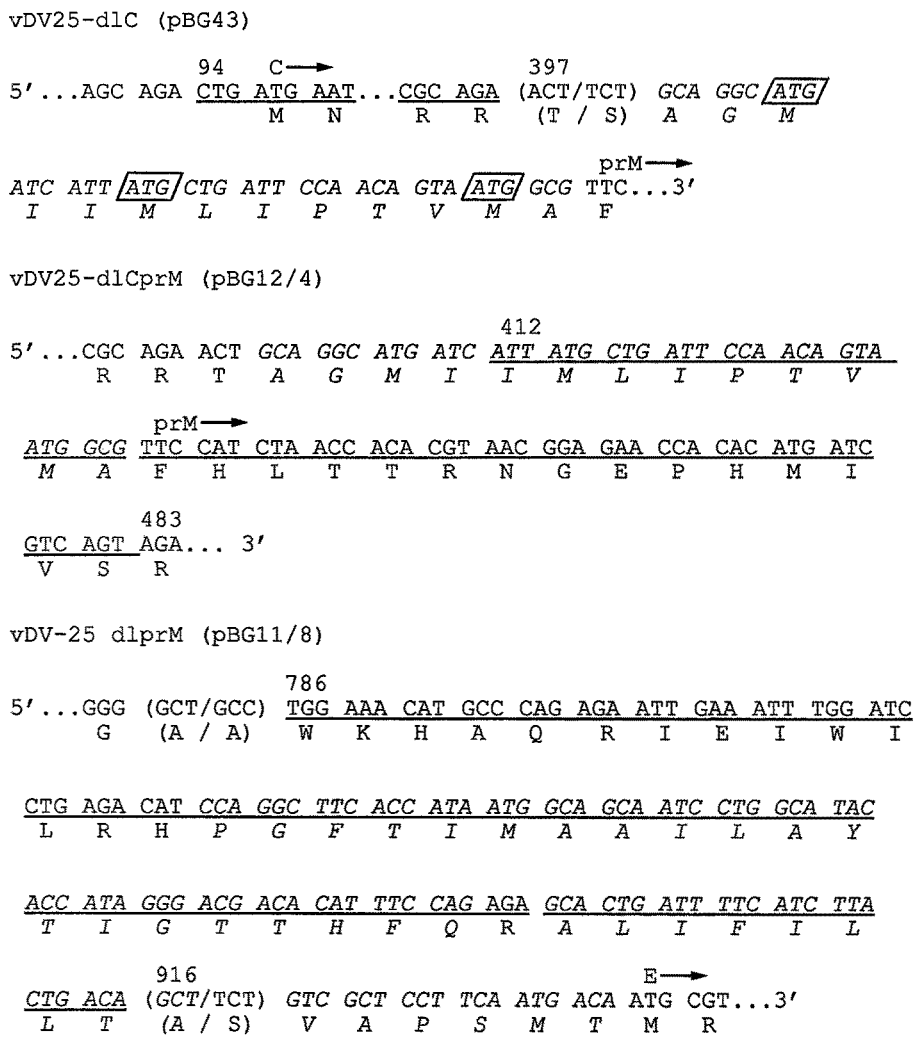

Fig. 2. Coding changes introduced into the DEN-2 sequence of each recombinant vaccinia virus. DEN-2 cDNA sequences are numbered according to Irie et al. [16]. Deleted sequences are underlined and those nucleotides encoding the hydrophobic domains are italicised. Where codons were altered rather than deleted, the original and new codons are shown in parentheses. For vDV25-dlC, possible start codons in frame with the DEN-2 ORF are boxed

Construction of recombinant vaccinia viruses

The protocols for generation of recombinant TK^- vaccinia viruses were based on methods described by Mackett et al. [19, 20]. Monolayers of L929 cells previously infected with WR strain vaccinia virus were transfected with calcium phosphate precipitates of the appropriate recombinant transfer plasmid DNA.

Individual plaques containing TK^- vaccinia viruses were isolated by growth on 143B cell monolayers in the presence of 25 μg/ml of BUdR. Recombinants were identified by DNA dot blot hybridization using a cDNA probe radiolabelled by the nick translation reaction [26], and plaque purified three times under selective pressure. Southern blotting of restriction digests of viral DNA followed by hybridization with a radiolabelled probe

was used to verify the presence of the required DEN-2 cDNA sequence in the recombinant vaccinia viruses.

Indirect immunofluorescent antibody staining

Coverslip cultures of 143 B cells (75% confluent) were infected with 10^3 PFU of wild type of recombinant vaccinia virus in 0.1 ml of inoculum. At 24 h, when distinct foci of infection or plaques were evident, the cells were fixed with ice-cold acetone and stained with 25 µl of antibody for 1 h at room temperature. The antibodies were monoclonal hybridoma fluids prepared by Dr. C. Parrish. Either a monoclonal antibody directed against the prM protein or a pool six monoclonal antibodies directed against the E glycoprotein were used. The cells were counterstained for 30 min with 25 µl of fluorescein-isothiocyanate (FITC) conjugated anti-mouse immunoglobulin raised in sheep (Silenius). The cells were examined by epi-illumination fluorescence microscopy using a Zeiss IM 35 inverted microscope (Carl Zeiss Pty. Ltd) and photographed using Kodak EPD-200 film.

Coverslip cultures of 143 B cells infected with DEN-2 were used as positive controls. Each culture was infected with 10^7 PFU of DEN-2 PUO-218 strain. At 48 h p.i., when CPE was 25–50%, the cells were fixed and stained as described for the vaccinia virus-infected cultures.

Preparation of radiolabelled infected cell lysates

To prepare labelled proteins for both wild type and recombinant vaccinia viruses, 143 B cells were infected at a moi of 2.0 PFU/cell. For DEN-2 virus, Vero cells were infected at a moi of 1.0 PFU/cell. Infected cell proteins were radiolabelled with [^{35}S]methionine essentially as described by Smith and Wright [30] except that actinomycin D was not included in the medium of vaccinia virus-infected cells. The starvation and labelling periods were for 1 h each, begun at 12 h p.i. for vaccinia virus-infected cells and at 72 h p.i. for DEN-2 infected cells. In some cases, a chase period of 12 h followed the pulse-labelling.

Radioimmunoprecipitation and electrophoresis of virus specified proteins

For each radioimmunoprecipitation (RIP), 100 µl of labelled cell lysate in RIP lysis buffer [30] was mixed with 10 µl of antiserum and incubated for 14–16 h at 4 °C with continuous end-over-end rotation. Immunoprecipitated antigens were recovered from solution by binding to protein-A coated Sepharose beads (Pharmacia) and finally the antigens were eluted from the beads by heating at 100 °C for 5 min in electrophoresis sample buffer [18]. The polyclonal antisera used were specific for the DEN-2 glycoproteins prM and E were raised in rabbits immunised with viral proteins eluted from polyacrylamide gels by Dr. G. W. Smith [30].

Samples prepared by RIP were analysed in 12 or 15% polyacrylamide gels [18]. A mixture of [^{14}C]methylated marker proteins (Amersham) was co-electrophoresed to allow estimation of molecular weights. After electrophoresis, the gels were treated for fluorography [3]. The radiolabelled proteins were detected by exposure of the gels to preflashed Kodak X-AR film for various length of time at − 70 °C.

Recovery of extracellular radiolabelled proteins from infected cells

Cells infected with DEN-2 or recombinant virus were radiolabelled as described above, including a chase of 12 h. Medium was recovered from infected cell monolayers and clarified by low speed centrifugation. The pellets were discarded, and supernatant fluids centrifuged at 50,000 rpm in a Beckman SW 56 rotor for 2 h at 4 °C. At this stage both the pellets and supernatant fluids were retained.

The pellets were resuspended in RIP lysis buffer. Approximately 6 ml of supernatant fluid was concentrated by repeated centrifugation through Centricon 30 microconcentrators (Amicon) until less than 0.1 ml of volume remained. Finally, 200 µl of the lysis buffer was added, the microconcentrators inverted, and residual material collected by a short low speed centrifugation. All extracellular samples were analysed in the same manner as described for cell lysate material.

Results

Detection of DEN-2 antigens in cells infected with recombinant vaccinia virus

Indirect immunofluorescence (IF) assays using a mixed pool of monoclonal antibodies indicated that all vaccinia virus recombinants produced E, or E-related proteins in the perinuclear region of infected cells (Figs. 3 D–G). The staining pattern was similar to that obtained for DEN-2 virus-infected cells (Fig. 3 B). The intensity of staining for vDV 25-dlCprM (Fig. 3 F) was less than for the other recombinants. Perinuclear staining was also a feature of IF assays using the anti-prM monoclonal antibody (Fig. 4). The staining of cells infected with vDV 25 (Fig. 4 D) and vDV 25-dlprM (Fig. 4 G) was weak, but relatively strong for vDV 25-dlC (Fig. 4 E). No staining at all was detected for vDV 25-dlCprM (Fig. 4 F).

DEN-2 polypeptides synthesized in cells infected with vDV25, vDV25-dlC, or vDV25-dlCprM

Analysis of vDV 25 virus-infected cells by RIP using a polyclonal antiserum specific for E revealed a unique polypeptide (Fig. 5 A, lane 3) that comigrated with E protein immunoprecipitated from DEN-2 virus-infected cells (Fig. 5 A, lane 5). Proteins with the mobility of E were also recovered by RIP of cells infected with vDV 25-dlCprM (Fig. 5 A, lane 1) and vDV 25-dlC (Fig. 5 A, lane 2). In the latter case a polypeptide of M_r 75,000–80,000 was also detected by the E antiserum.

The same radiolabelled cell lysates used in the experiment shown in Fig. 5 A were immunoprecipitated with a polyclonal antiserum directed against prM (Fig. 5 B). A protein comigrating with DEN-2 prM (Fig. 5 B, lane 5) was detected only in vDV 25-dlC virus-infected cells (Fig. 5 B, lane 2); no such protein was observed for vDV 25 (Fig. 5 B, lane 3). Deubel et al. [11] also failed to detect prM using a similar construct of DEN-2 cDNA in vaccinia virus. However, immunoprecipitation of the vDV 25 lysate by antiserum raised against a bacterial fusion protein containing the non-M portion of prM revealed a faint band comigrating with prM (Murray, unpubl. results). Polypeptides of M_r 33,000 (Fig. 5 B, lanes 1–3), and M_r 22,000 (Fig. 5 B, lanes 1 and 2) were not present in cells infected by WR or DEN-2 (Fig. 5 B, lanes 4 and 5). These proteins were not consistently observed in other gels. It is possible that the first corresponds to C + prM (applicable to vDV 25 only), and the second to a polypeptide observed in DEN-2 virus-infected cells following short pulses of radioactive label [30].

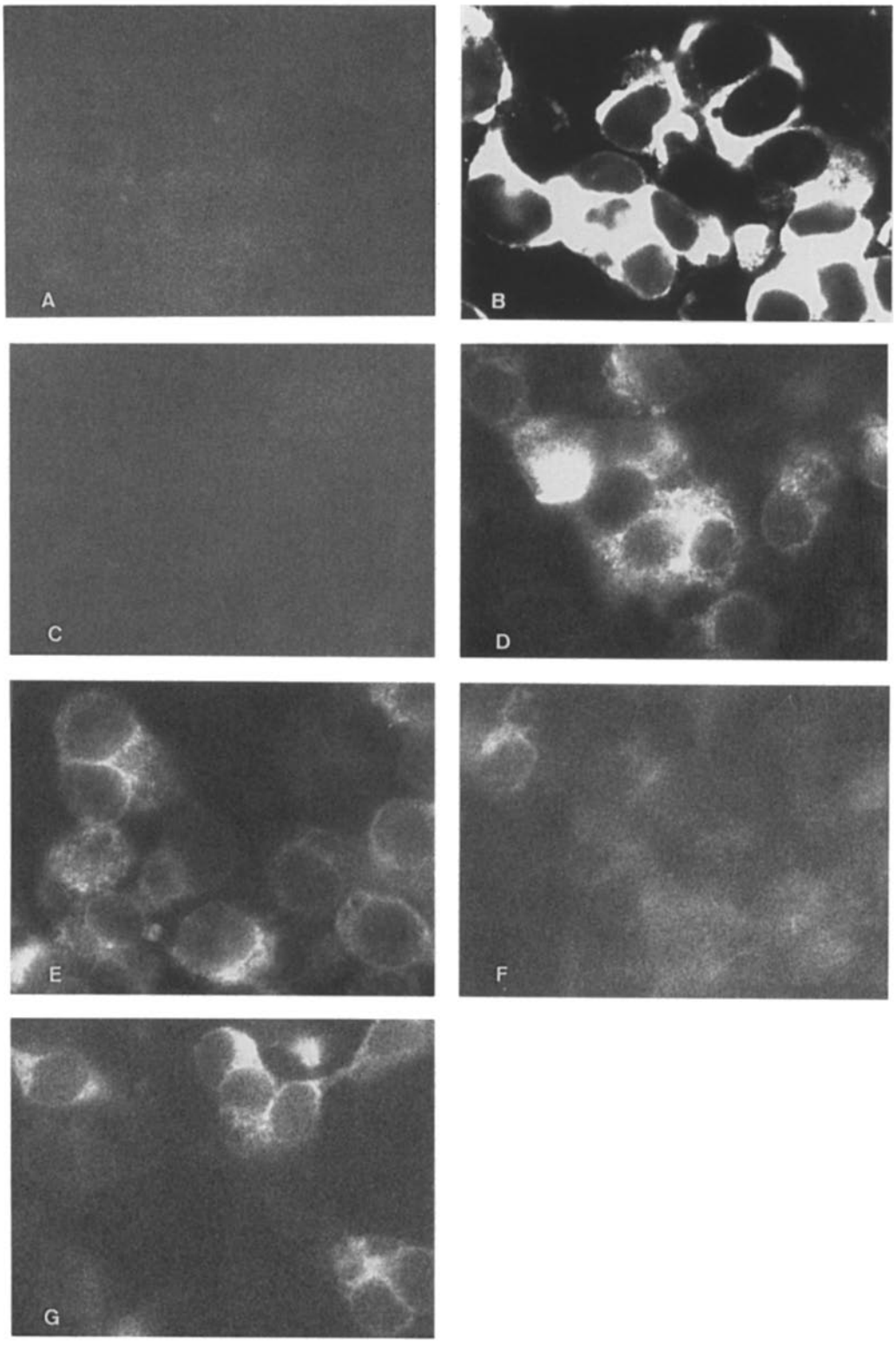

Fig. 3. Detection of DEN-2 E protein in infected cells by indirect immunofluorescence using a pool of anti-E monoclonal antibodies. Cells were **A** mock infected, infected with **B** DEN-2 virus, **C** WR vaccinia, **D** vDV 25, **E** vDV 25-dlC, **F** vDV 25-dlCprM, or **G** vDV 25-dlprM

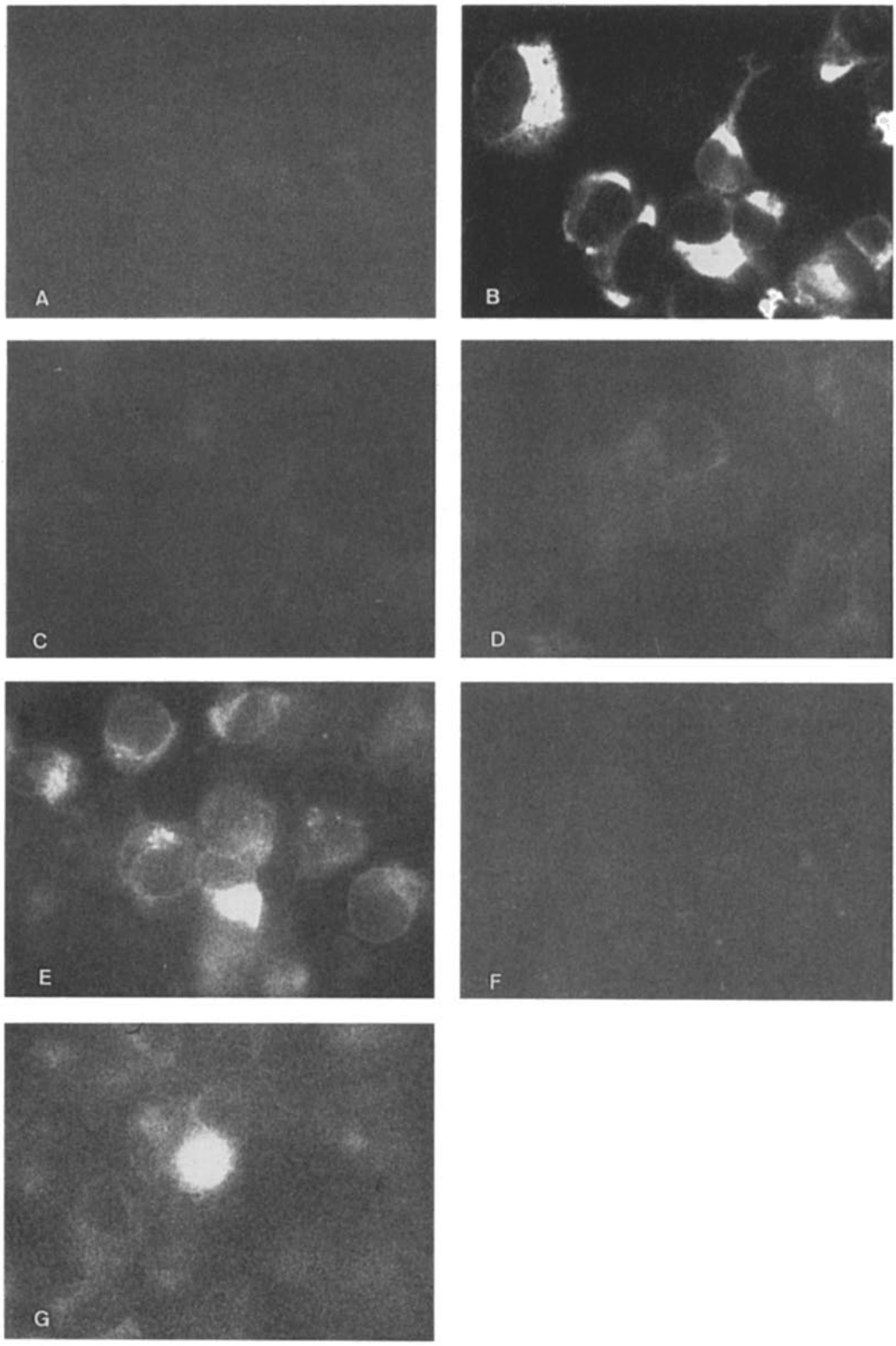

Fig. 4. Detection of DEN-2 prM protein in infected cells by indirect immunofluorescence using an anti-prM monoclonal antibody. Cells were **A** mock infected, infected with **B** DEN-2 virus, **C** WR vaccinia, **D** vDV 25, **E** vDV 25-dlC, **F** vDV 25-dlCprM, and **G** vDV 25-dlprM

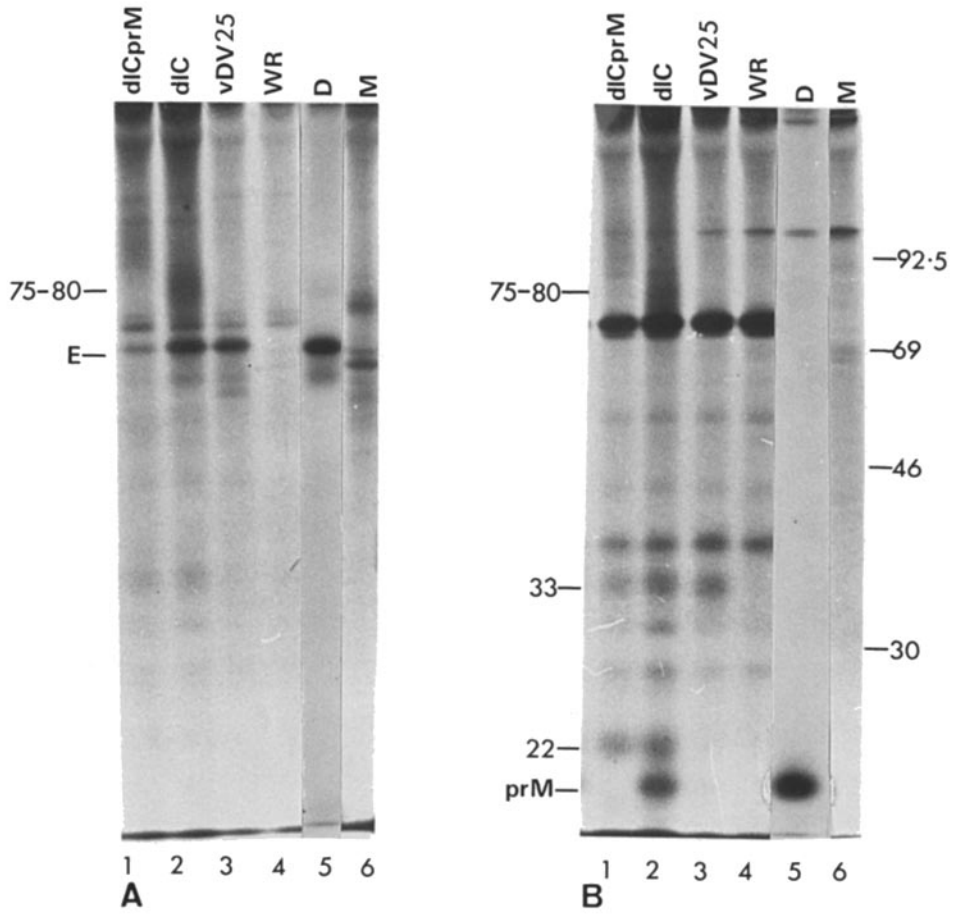

Fig. 5. Analyses in a 12% polyacrylamide gel of immunoprecipitates of [^{35}S]methionine-labelled proteins from infected cell lysates. Antisera were directed against **A** DEN-2 E protein, **B** DEN-2 prM protein. Equal volumes of immunoprecipitated cell lysates were loaded for each lane. Cells were infected with vDV 25-dlCprM (*1*), vDV 25-dlC (*2*), vDV 25 (*3*), WR vaccinia (*4*), DEN-2 virus (*5*), or mock infected (*6*)

The RIP results correlated well with those obtained by IF. In particular, the deletion of the prM signal sequence and the prM amino terminus (vDV 25-dlCprM) apparently prevented synthesis of correctly processed and stable prM, but not E. This demonstrated that the E protein signal sequence acted independently of the signal sequence preceding prM consistent with the results obtained by translation in vitro of yellow fever virus RNA [27]. In addition less E was detected in cells infected by vDV 25-dlCprM than in those infected by vDV 25, suggesting that prM may have some additional role in the processing or stability of prM. It is also clear by both RIP and IF of vDV 25-dlC virus-infected cells that 11 (or perhaps fewer) amino acids at the carboxy terminus of C were sufficient for the formation of correctly processed prM and E. Deletion of almost 90% of C had no apparent adverse effect on their synthesis. Fur-

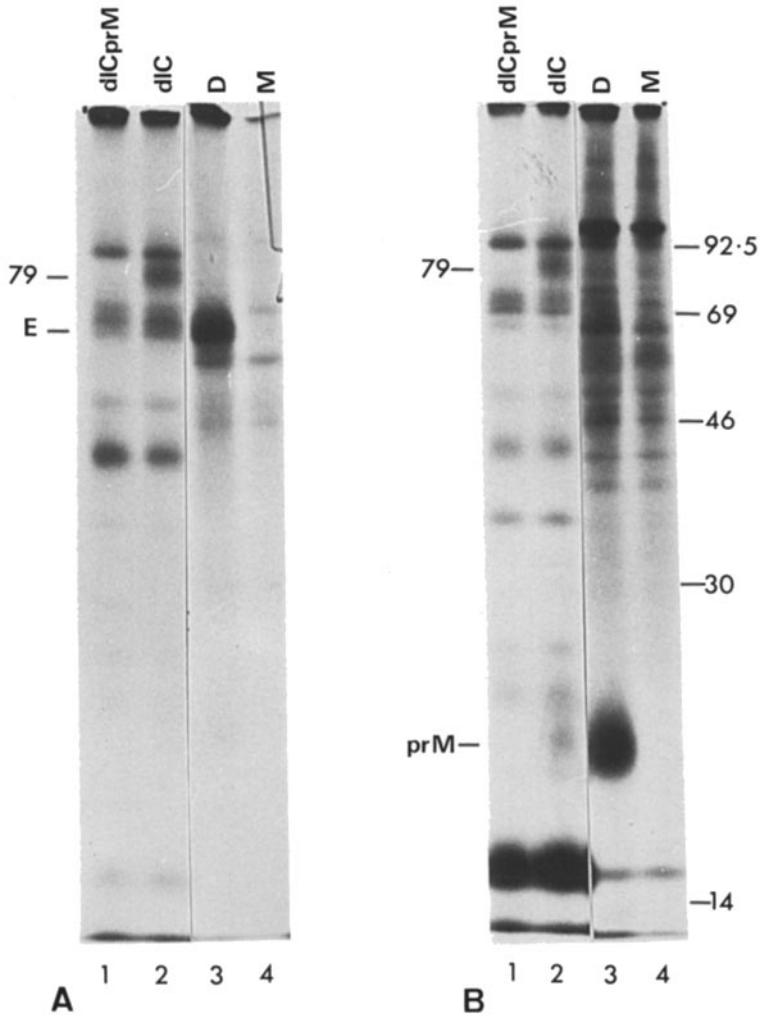

Fig. 6. Analyses in a 14% polyacrylamide gel of [^{35}S]methionine-labelled proteins immunoprecipitated with antiserum directed against **A** DEN-2 E protein or **B** prM protein. Cells were infected with vDV 25-dlCprM (*1*), vDV 25-dlC (*2*), DEN-2 (*3*), or mock infected (*4*)

thermore, initiation of protein synthesis occurred at one or more of the potential start codons indicated in Fig. 2.

The protein of M_r 75,000–80,000 precipitated by the anti-E and anti-prM antisera from cells infected by vDV 25-dlC (Fig. 5 A and B, lane 2) corresponded in size to that predicted for an uncleaved polypeptide of prM and E. The protein was better resolved by electrophoresis through a more concentrated polyacrylamide gel (Fig. 6). Precipitation of the protein using antiserum directed against either E (Fig. 6 A, lane 2) or prM (Fig. 6 B, lane 2) was equally efficient. The sharper bands obtained allowed a more precise estimation of the size as M_r 79,000.

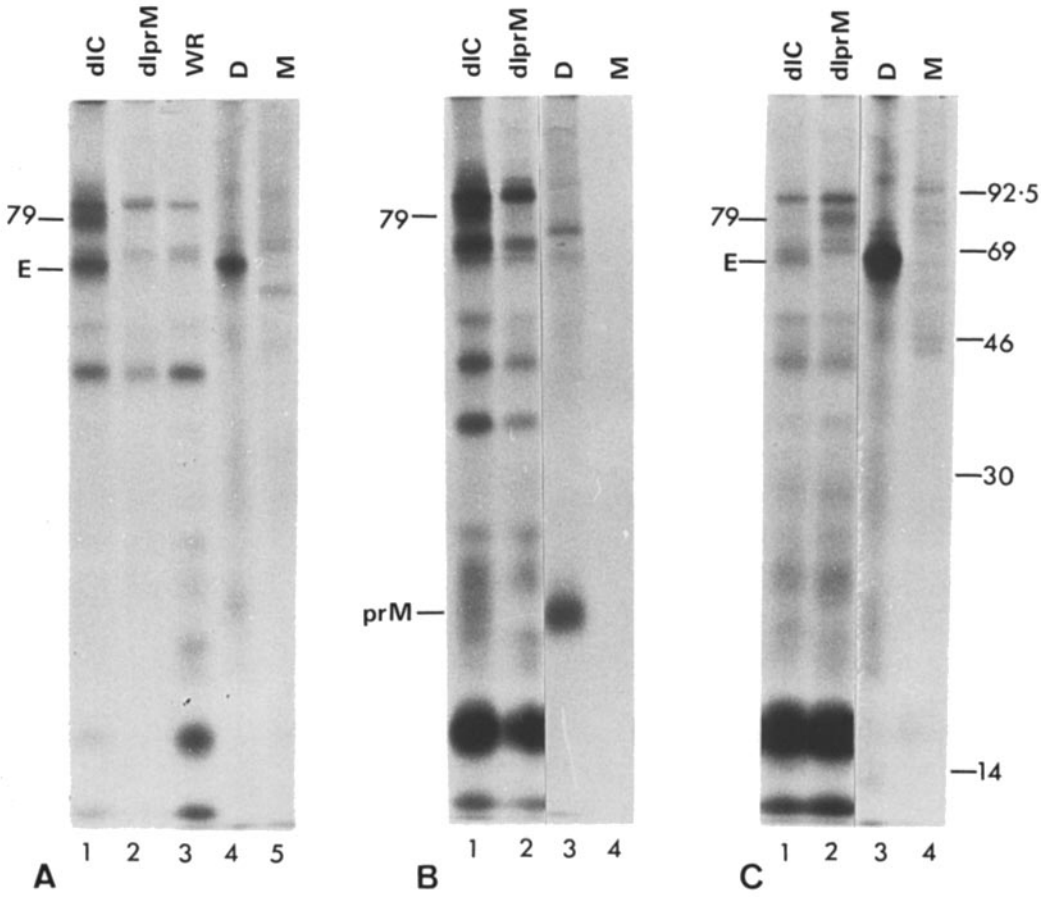

Fig. 7. Analyses in a 14% polyacrylamide gel of [^{35}S]methionine-labelled proteins immunoprecipitated from infected cell lysates. Cells were either pulse-labelled for 1 h (**A** and **B**), or pulse-labelled and then chased for 12 h (**C**). Proteins were precipitated either by E specific polyclonal antiserum (**A** and **C**), or by prM antiserum (**B**). **A** Cells were infected with vDV 25-dlC (*1*), vDV 25-dlprM (*2*), WR vaccinia (*3*), DEN-2 (*4*), or mock infected (*5*). **B** and **C** Cells were infected with vDV 25-dlC (*1*), vDV 25-dlprM (*2*), DEN-2 (*3*), mock infected (*4*)

DEN-2 polypeptides synthesised in cells infected with vDV 25-dlprM

The recombinant vDV 25-dlprM was constructed to examine the effect of protein processing of a deletion encoding most of the hydrophobic residues at the carboxy terminus of prM. When cells infected by this virus were pulse-labelled for 1 h, no unique proteins comigrating with authentic DEN-2 proteins were detected (Fig. 7 A and B, lane 2). However, perinuclear staining by IF using monoclonal antibodies reactive with prM or E (Figs. 3 G and 4 G) indicated the presence of DEN-2 antigens. In a further attempt to detect correctly processed DEN-2 proteins, cells were infected with vDV 25-dlprM, labelled for 1 h and then chased for 12 h. Analysis of the cell lysate by RIP using antiserum

directed against E revealed a protein of M_r 79,000 (Fig. 7 C, lane 2). There was no evidence of a protein comigrating with authentic DEN-2 E.

Similarly, immunoprecipitates of lysates of cells infected with vDV 25-dlC and pulse-labelled (Fig. 7 A and B, lane 1) were compared with those prepared from cells following a chase (Fig. 7 C, lane 1). In these experiments, a protein of M_r 79,000 was detected after the pulse (see also Fig. 6 A and B, lane 2), but not following the chase. However, the band corresponding to E remained after the chase indicating the stability of this protein.

Detection of extracellular E protein

Since cells infected by vDV 25 and vDV 25-dlC viruses contained both E and prM in correctly processed forms, it was of interest to determine whether particles such as slowly sedimenting haemagglutinating particles (SHA), were released from cells in a manner analogous to the release of DEN-2 SHA and virions from DEN-2 virus-infected cells. Thus the polypeptide composition of the culture medium from cells infected with vDV 25 or vDV 25-dlC was analysed. The medium was recovered, and processed as described in the Methods. Samples of both the pellet and supernatant fluid following ultracentrifugation were immunoprecipitated with the polyclonal antiserum directed against E (Fig. 8).

Almost all immunoprecipitated material for vDV 25 was present in the supernatant fluid (compare lanes 2 in Fig. 8 A and B) unlike the DEN-2 virus control (lane 4). This result suggested that the majority of E molecules released from cells infected with vDV 25 were in a soluble form, and not assembled into sedimentable particles. The E released by vDV 25 virus-infected cells was confined to a single band estimated at M_r 3,000 smaller than authentic DEN-2 E protein.

The culture medium of cells infected with vDV 25-dlC was analysed in a similar manner. However, in this case most of the immunoprecipitated material was contained in the pellet after ultracentrifugation (Fig. 8 B, lane 1), and was detected as a band comigrating with authentic DEN-2 E protein (lane 4). This result suggested that E protein released from cells infected with vDV 25-dlC was correctly processed and was associated with sedimentable particles. Recently, haemagglutinating activity (HA) was reported in the medium of cells infected with a vaccinia virus containing a Japanese encephalitis virus (JE) construct similar to vDV 25-dlC, but also encoding NS 1 and NS2A [22]. The HA activity comigrated with JE SHA particles in sucrose gradients. Our result may indicate the production by vDV 25-dlC of similar particles for DEN-2.

Discussion

The glycoproteins prM (M_r 20,000) and E (M_r 60,000) were synthesised, cleaved and glycosylated in cells infected by vDV 25-dlC. This conclusion is based on comparisons of their mobilities in gels with the mobilities of corresponding proteins in DEN-2 virus-infected cells. Additional experiments using tunica-

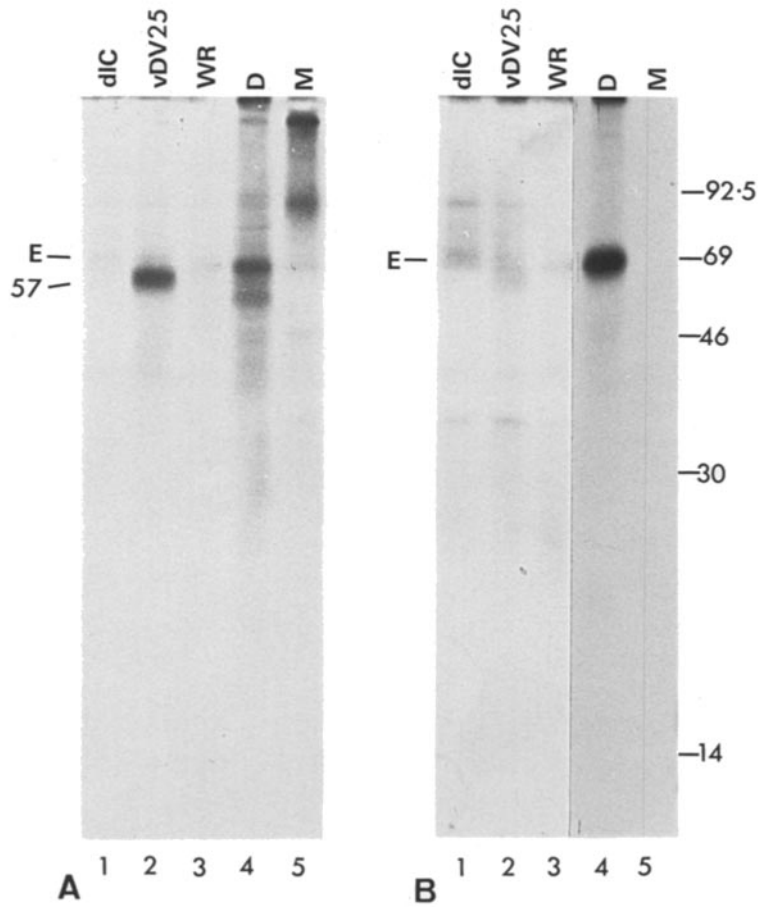

Fig. 8. Analyses in a 15% polyacrylamide gel of [^{35}S]methionine-labelled extracellular proteins immunoprecipitated by antiserum directed against E. Medium from cells which were pulse-labelled for 1 h and chased for 12 h was analysed. **A** Samples prepared from material in medium which failed to pellet after ultracentrifugation; **B** samples prepared from material which pelleted. The cells were infected with vDV 25-dlC (*1*), vDV 25 (*2*), WR vaccinia (*3*), DEN-2 (*4*), or mock infected (*5*)

mycin confirmed that they were glycosylated (data not shown). Since the amino terminal 90% of C is not required for the correct processing of prM and E, it is apparent that (*i*) the hydrophobic domain in the middle of C (amino acids 56 to 66) does not have an essential role in the attachment of nascent polyprotein to the RER and (*ii*) C is not the proteinase which cleaves at the amino terminus of prM, E and NS 1. These findings are in agreement with those obtained by cell-free translation of RNA transcripts of yellow fever and dengue virus type 4 (DEN-4) cDNA [21, 27] and expression of JE cDNA using vaccinia virus [22].

Unexpectedly, prM was more readily detected in cells infected with vDV 25-dlC than in those infected with vDV 25 (Figs. 4 E and 5 B). There are several potential initiation codons in vDV 25-dlC which are shown in Fig. 2. Initiation

at the first, second and third of these would produce hydrophobic sequences of 11, 8, and 2 amino acids, respectively, immediately preceding the amino terminus of prM. All are under the minimum length of 13 amino acids suggested for a signal sequence by von Heijne [34]. The third AUG codon is in the most favoured context compared with the other two for the initiation of translation [17]. Our results suggested that more than one of the codons was utilised. The detection of glycosylated prM and E indicated the presence of a functioning signal sequence, most probably because initiation occurred at the first AUG. The protein of M_r 79,000 may have arisen in two ways, either by lack of cleavage at the site between prM and E which were otherwise correctly processed, or by the generation of an untranslocated and unglycosylated molecule beginning at one of the three possible AUG codons and terminating at the stop codon in the pUC 8 sequences. The second possibility would most likely arise through usage of the second or third AUG codons. Experiments using tunicamycin (not shown) were inconclusive in determining whether the M_r 79,000 protein was glycosylated. Usually it migrated as a broad band through gels, and thus was possibly a mixture of molecules arising from initiation events at the alternative codons. Its disappearance during the chase (Fig. 7 C) may have been due to degradation of the untranslocated and unglycosylated full-length product, or slow cleavage of glycosylated prM and E. Our experiments did not distinguish between the two alternatives.

For vDV 25-dlCprM the internal deletion removed nearly 65% of the signal sequence preceding prM, including the signal peptidase cleavage site, and 15 amino acids at the amino terminus of prM. The results showed that the deletion did not prevent the glycosylation of E and its cleavage from the polyprotein. Thus the hydrophobic domain immediately preceding the amino terminus of E acted independently as an internal signal sequence; prior insertion of prM into the membrane was not necessary for its function. Similar results were obtained by cell-free translation of yellow fever RNA [27]. The failure to detect truncated prM or prM-related proteins (e.g. C + prM) in cells infected by recombinant vaccinia virus (Figs. 4 F and 5 B) suggested that such proteins were unstable.

The deletion in the final recombinant virus vDV 25-dlprM removed 43 amino acids towards the carboxy terminus of prM. Six hydrophobic amino acids immediately preceding the amino terminus of E were retained. The construct was designed to test whether the signal sequence preceding prM was sufficient for the translocation of both prM and E, and whether signal peptidase would cleave at the amino terminus of E in absence of all but six amino acids of signal sequence. Since no prM and E were detected in cells infected by vDV 25-dlprM (Fig. 7), we concluded that cleavage at their junction did not occur. After the chase (Fig. 7 C, lane 2), a protein of M_r 79,000 was detected. This corresponded in size to a fully glycosylated protein of prM + E. Such a protein may have formed by delayed cleavage at the C-prM junction of a glycosylated polyprotein C + prM + E (estimated M_r 92,000), unresolved in our gels because of comi-

grating host of vaccinia polypeptides (Fig. 7 A, lanes 1 to 3). Thus the formation of the polypeptide of M_r 79,000 suggested that the signal sequence for prM was sufficient for the insertion of prM and E into the RER, and that signal peptidase cleaved at the C-prM junction but not at the modified prM-E junction.

Expression of correctly processed extracellular prM and E by recombinant vaccinia viruses containing JE cDNA sequences correlated with the detection of SHA activity and the induction of protective and neutralizing antibodies [22]. In previous studies on the expression of flavivirus genes in vaccinia, no extracellular structural proteins were detected. Neutralising antibodies were either not induced at all [6, 39], or were detected at low titres in only a few of the animals immunized [15]. Mason and his colleagues [22] have suggested that a requirement for the formation of extracellular particles containing prM and E is the presence of the genes for all three flavivirus glycoproteins in the construct. Further, they suggest that the C protein encoded in the recombinant genomes used by previous workers, but not by their own constructs, may interfere with the proper assembly of prM and E in this system.

We have now demonstrated the release of E from cells infected by vDV 25 which encodes all of the DEN-2 structural proteins, including C (Fig. 8 A, lane 2). However, this protein did not comigrate with the authentic extracellular DEN-2 E protein (Fig. 8 A, lane 4), was apparently in a soluble form, and was therefore unlikely to represent assembled SHA-like particles. The composition of the soluble E protein is under investigation. E of the correct size and in a form which did sediment, was recovered from the medium of cells infected with vDV 25-dlC (Fig. 8 B, lane 1). Since the construct encoded prM and E, but not NS 1, these preliminary results suggested that only prM and E may be needed for the synthesis of extracellular DEN-2 SHA-like particles by recombinant vaccinia virus. Further work to confirm the presence of such particles and to characterize their structure and composition is currently in progress.

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