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Expression of Dengue Virus Structural Proteins and Nonstructural Protein NS_1 by a Recombinant Vaccinia Virus

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A recombinant vaccinia virus containing cloned DNA sequences coding for the three structural proteins and nonstructural proteins NS_1 and NS_{2a} of dengue type 4 virus was constructed. Infection of CV-1 cells with this recombinant virus produced dengue virus structural proteins as well as the nonstructural protein NS_1. These proteins were precipitated by specific antisera and exhibited the same molecular size and glycosylation patterns as authentic dengue virus proteins. Infection of cotton rats with the recombinant virus induced NS_1 antibodies in 1 of 11 animals. However, an immune response to the PreM and E glycoproteins was not detected. A reduced level of gene expression was probably the reason for the limited serologic response to these dengue virus antigens.

Infections by dengue virus types 1 through 4 continue to pose a major public health problem in many geographic areas, especially in Southeast Asia, the South Pacific, the Caribbean, and Central and South America. Dengue virus infections also occasionally cause hemorrhagic fever with a shock syndrome which has a high mortality rate in children and young adults (4, 10). Unfortunately, there is a lack of specific immunoprophylactic measures against dengue virus, and prevention of dengue virus infection still depends on mosquito vector control, which is labor intensive and often ineffective.

Like other members of the flavivirus family, extracellular dengue virus has a relatively simple structure. Virions contain only three virus-coded proteins, designated capsid (C) protein, membrane (M) protein, and envelope (E) glycoprotein (3, 6, 14). Intracellular virus, which is also infectious, lacks M but contains another glycoprotein, PreM, from which M is derived by cleavage (11). Both C and M are internal proteins. The surface envelope glycoprotein is the major site responsible for neutralization of infectivity by specific antibodies (12, 13). The envelope glycoprotein also exhibits hemagglutinating activity and is responsible for adsorption to the cell surface (16). Several nonstructural proteins have also been identified in dengue virus-infected cells. Among them is nonstructural protein NS_1, which is a glycoprotein and was originally described as a soluble complement-fixing antigen. NS_1 may also play an important role in mediating immunity, since the analogous NS_1 glycoprotein of yellow fever virus has been shown to be a protective antigen in mice and in primates (7, 8). Recently, the NS_1 of dengue type 2 virus was also shown to be a protective antigen (9).

The dengue type 4 virus genome consists of a molecule of positive-stranded RNA 10,644 nucleotides in length (15). A full-length cDNA copy of the dengue type 4 virus genome was recently prepared, and its complete nucleotide sequence was determined (5, 18). This analysis indicated that 96% of the dengue virus genome codes for a polyprotein which is apparently cleaved by a specific protease(s) to generate individual viral proteins (2). The three structural proteins, C-M-E, are located at the amino terminus, while nonstructural proteins NS_1-NS_{2a}, NS_{2b}-NS_3-NS_{4a}, NS_{4b}, and NS_5 are at the carboxy terminus (5, 18). Because dengue virus gene expression involves proteolytic cleavage of the polyprotein, we attempted to achieve expression of protective antigens E and NS_1 from their cloned DNA sequences for evaluation in experimental immunoprophylaxis. Vaccinia virus was used as a vector for construction of a live recombinant virus. The *Bgl*II DNA fragment (4,040 base pairs, nucleotides 88 to 4128) from the 5′ terminus of dengue type 4 virus cDNA contains the coding region for the three structural proteins as well as nonstructural proteins NS_1 and NS_{2a}. This fragment was excised from the full-length dengue virus DNA copy and was inserted into the PSC11 vaccinia virus intermediate vector (1). The dengue virus DNA sequence was inserted into the *Bam*HI site immediately downstream of the vaccinia P7.5 early-late promoter (Fig. 1). In this construct, the dengue virus coding sequence was placed under the transcriptional control of the vaccinia virus P7.5 early-late promoter. The vector contained a bacterial β-galactosidase gene under the control of the vaccinia virus P11 late promoter, which provided a visual selectable marker. The chimeric genes were flanked by sequences of the vaccinia virus thymidine kinase gene, which directed homologous recombination of dengue virus sequences into the vaccinia virus genome following transfection of CV-1 cells previously infected with wild-type vaccinia virus (WR strain). Recombinant vaccinia virus harboring the dengue virus DNA insert was isolated and plaque purified two times on TK^- cells in selective medium.

Dengue virus-specific proteins synthesized during infection with the recombinant virus were initially detected by indirect immunofluorescence. CV-1 cells infected with the recombinant virus exhibited fluorescence-stainable antigens in the cytoplasm when polyvalent dengue type 4 virus hyperimmune mouse antiserum was used, although the intensity of staining was less than that observed in dengue virus-infected cells. Similar immunofluorescence was observed in CV-1 cells infected with the recombinant virus when monoclonal antibody specific to the E glycoprotein

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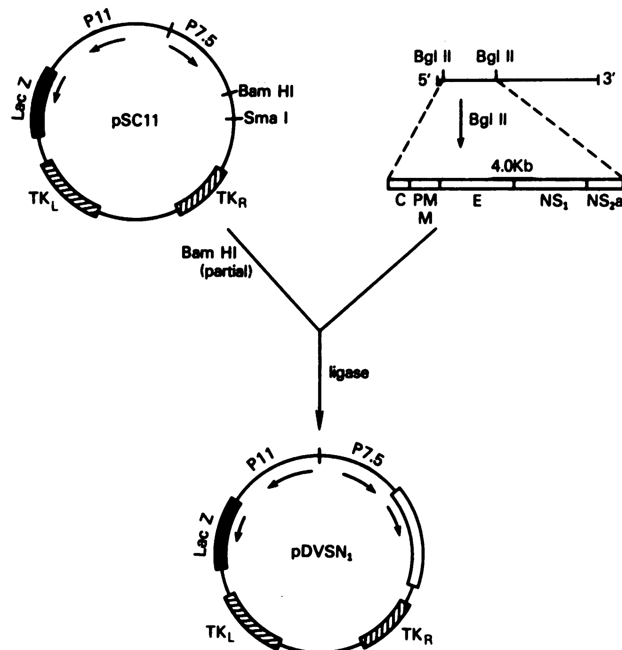

FIG. 1. Construction of dengue virus-vaccinia virus recombinant plasmid. The intermediate cloning vector psc11 contains interrupted thymidine kinase gene sequences (TK_R and TK_L) and a bacterial β-galactosidase gene (LacZ) under the transcription control of the P11 vaccinia virus promoter. The vector DNA was partially digested with *Bam*HI to open the *Bam*HI site downstream of the P7.5 promoter for insertion of the 4.1 kilobase (Kb) fragment of dengue virus DNA. Recombinant plasmid containing the dengue virus DNA insert in the sense transcription orientation was selected and used for isolation of recombinant vaccinia virus.

was used. To further identify this and other dengue virus proteins, recombinant virus-infected cells were radiolabeled with [^{35}S]methionine and the cell lysate was prepared for immunoprecipitation by specific antibodies. Analysis of the labeled precipitate on sodium dodecyl sulfate-polyacrylamide gel (Fig. 2A) showed that the polyvalent hyperimmune mouse antiserum precipitated three major bands with estimated molecular sizes of 20, 40 to 46, and 55 to 60 kilodaltons (kDa), respectively. The minor bands of larger molecular size were probably nonspecific. When an E-glycoprotein-specific monoclonal antibody (1H10) was used, a 55 to 60-kDa band consistent with the molecular size of the E glycoprotein was precipitated. Similarly, dengue type 2 virus NS_1-specific antiserum (kindly supplied by J. Schlesinger) precipitated a 40- to 46-kDa band, which is the predicted size of the NS_1 nonstructural glycoprotein. The third major band, of approximately 20 kDa, precipitated by dengue type 4 virus hyperimmune antiserum and consistent with the intracellular PreM glycoprotein in size, was also precipitated by PreM-specific antibodies (monoclonal antibody 2H2 or 5C9, kindly provided by M. K. Gentry). Thus, in recombinant virus-infected cells, the three glycoproteins encoded in the cloned DNA appeared to be cleaved and modified by glycosylation in a manner similar to that observed during dengue virus infection. This suggests that the dengue virus structural proteins, as well as NS_1 nonstructural protein, were specifically processed by proteolytic cleavage of the polyprotein in the absence of dengue virus functions provided by the distal nonstructural proteins NS_{2b} through NS_5. However, the amount of dengue virus PreM, E, and NS_1 glycoproteins produced in recombinant virus-infected cells was significantly less than that produced in dengue type 4 virus-infected cells, as estimated by immunoprecipitation.

The pattern of glycosylation of the glycoproteins produced in recombinant virus-infected cells varied as indicated by their response to endoglycosidase H, which cleaves the mannose-rich carbohydrate core (17). The PreM protein band was completely sensitive to endoglycosidase H treatment, yielding a band of 17 kDa, a reduction of 3 kDa in molecular size (Fig. 2B). On the other hand, a significant portion of the carbohydrate of both the E and NS_1 glycoproteins appeared to be resistant to endoglycosidase H digestion.

Each of the dengue virus glycoproteins, i.e., PreM, E, and NS_1, is preceded by a stretch of hydrophobic amino acids which can serve as a signal, and hence cellular signalase is most likely responsible for proteolytic cleavage. Presumably, the cleavage mechanism that generated the three glycoproteins also yielded the capsid protein, which is located amino terminal to the PreM glycoprotein. On the other hand, the processed product of PreM, M protein, could not be identified with certainty in recombinant vaccinia virus-infected cells.

The recombinant vaccinia virus was used for immunization of cotton rats, and their immune responses were evaluated by serologic analysis. Cotton rats (six each of *Sigmodon hispidus* and *Sigmodon fulviventor*) were inoculated intradermally with 10^8 PFU of recombinant vaccinia virus. Control animals received the same dose of wild-type vaccinia virus (strain WR) or only phosphate-buffered saline. Serum samples collected 3 and 6 weeks after immunization were analyzed for the presence of dengue virus-specific antibodies by enzyme-linked immunosorbent assay, plaque reduction virus neutralization, and hemagglutination inhibition. None of these tests detected a dengue virus-specific response. However, all the animals developed a high titer of neutralizing antibodies against vaccinia virus (1:1,000 to 1:5,000), indicating that they had been infected with the recombinant virus.

Radioimmunoprecipitation with ^{35}S-labeled dengue virus proteins was also performed in an attempt to detect low levels of dengue virus-specific antibodies. NS_1 glycoprotein was precipitated by the serum of one animal at 6 weeks after immunization (Fig. 3). However, the same antiserum did not precipitate either the E or PreM glycoprotein. Sera from 10 other animals were all negative by this assay. Thus, the recombinant vaccinia virus failed to elicit a detectable seroresponse to the E or PreM glycoprotein.

There are several factors which may have contributed to the poor immune response to the NS_1 protein as well as to our failure to detect a seroresponse to the E glycoprotein. Most important was the low level of expression of dengue virus structural proteins and NS_1 by the vaccinia recombinant virus. The amount of dengue virus proteins produced in cells infected with the recombinant virus was approximately 1/10 that produced in cells infected with dengue type 4 virus. Translation of the dengue virus chimeric gene transcript from the recombinant virus may not have been optimal, because a region of the 5′ noncoding sequence not included in the recombinant virus might be required for efficient translation of the dengue virus polyprotein. Expression of dengue virus proteins by the vaccinia recombinant virus may also have been reduced, because optimal processing of the polyprotein may require participation of proteins produced by the downstream nonstructural genes not included in the

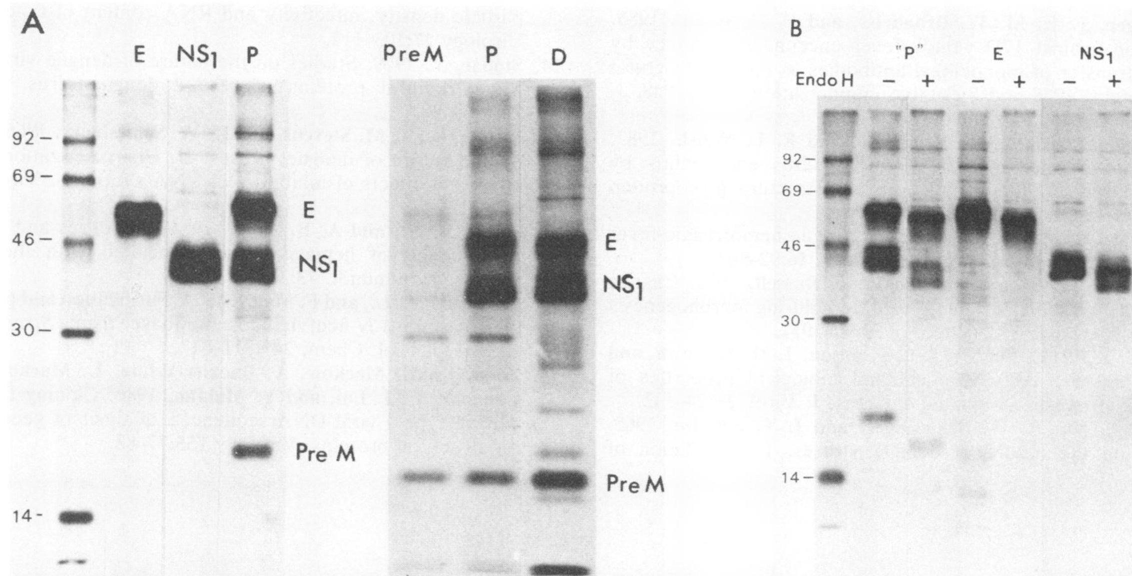

FIG. 2. Identification of dengue virus proteins synthesized by a recombinant vaccinia virus. (A) Immunoprecipitation of [^{35}S]methionine-labeled lysate from recombinant vaccinia virus-infected cells (multiplicity of infection, 10 PFU per cell) was carried out with one of the following specific antibodies: monoclonal antibody 1H10, specific for the envelope glycoprotein (E); rabbit antibodies prepared against dengue virus type 2 nonstructural protein 1 (NS_1); monoclonal antibody 5C9 specific for the membrane glycoprotein precursor (PreM); and polyvalent antibodies (P). The labeled precipitates were analyzed on sodium dodecyl sulfate–12% polyacrylamide gels. Also shown are the labeled dengue virus protein markers (D) obtained by immunoprecipitation with polyvalent antibodies of [^{35}S]methionine-labeled lysate from dengue virus-infected CV-1 cells. (B) Labeled immunoprecipitates prepared as described above were analyzed by digestion with endoglycosidase H (endo H) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Numbers at the left of the gels indicate protein sizes in kilodaltons.

recombinant virus. In addition, the E protein accumulated only intracellularly, and the NS_1 nonstructural protein was not expressed abundantly on the cell surface. These factors can now be explored by constructing vaccinia virus recombinants which include more of the 5′ noncoding region of

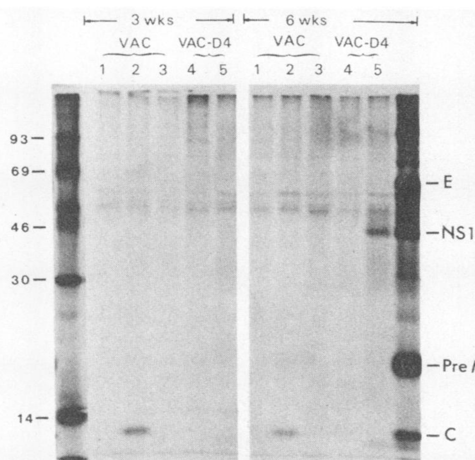

FIG. 3. Serologic response in cotton rats after immunization with recombinant vaccinia virus. Cotton rats were inoculated intradermally with 10^8 PFU of vaccinia virus (WR strain) carrying a β-galactosidase gene (VAC) or vaccinia virus-dengue virus recombinant virus (VAC-D4). Immunized animals were bled at 3 weeks and at 6 weeks. Serum samples were analyzed by radioimmunoprecipitation of [^{35}S]methionine-labeled antigens from dengue virus-infected cells. Serum samples were from three animals immunized with VAC and two animals immunized with VAC-D4. Dengue virus proteins and molecular size markers are also shown.

dengue virus or the downstream 3′ sequences. Other vaccinia virus promoters will be examined in an attempt to increase the level of expression of dengue virus structural proteins and NS_1.

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LITERATURE CITED

1. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
2. **Cleaves, G. R.** 1985. Identification of dengue type 2 virus specific high molecular weight proteins in virus-infected BHK cells. J. Gen. Virol. **66**:2767–2771.
3. **Deubel, J., R. M. Kinney, and D. W. Trent.** 1986. Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype. Virology **155**:365–377.
4. **Halstead, S. B.** 1980. Dengue haemorrhagic fever: a public health problem and a field for research. Bull. W.H.O. **58**:1–21.
5. **Mackow, E., Y. Makino, B. Zhao, Y.-M. Zhang, L. Markoff, A. Buckler-White, M. Guiler, R. Chanock, and C.-J. Lai.** 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for structural proteins. Virology **159**:217–228.
6. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evaluation. Science **229**:726–733.
7. **Schlesinger, J. J., M. W. Brandriss, C. B. Cropp, and T. P. Monath.** 1986. Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. J. Virol. **60**:1153–1155.

8. **Schlesinger, J. J., M. W. Brandriss, and E. E. Walsh.** 1985. Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein GP48 and by active immunization with GP48. J. Immunol. **135:**2805–2809.
9. **Schlesinger, J. J., M. W. Brandriss, and E. E. Walsh.** 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue virus non-structural glycoprotein NS1. J. Gen. Virol. **68:**853–857.
10. **Schlesinger, R. W.** 1977. Secondary dengue hemorrhagic fever and the shock syndrome. Virol. Monogr. **16:**92–104.
11. **Shapiro, D., W. E. Brandt, and P. K. Russell.** 1972. Change involving a viral membrane glycoprotein during morphogenesis of group B arboviruses. Virology **50:**906–911.
12. **Smith, T. J., W. E. Brandt, J. L. Swanson, J. M. McCown, and E. L. Buescher.** 1970. Physical and biological properties of dengue-2 virus and associated antigens. J. Virol. **5:**524–532.
13. **Stevens, T. M., R. W. Schlesinger, and I. T. Schulze.** 1965. Studies on the nature of dengue viruses. I. Correlation of particle density, infectivity and RNA content of type 2 virus. Virology **27:**103–112.
14. **Stollar, V.** 1969. Studies on the nature of dengue viruses. IV. The structural proteins of type 2 dengue virus. Virology **39:**426–438.
15. **Stollar, V., T. M. Stevens, and R. W. Schlesinger.** 1966. Studies on the nature of dengue viruses. II. Characterization of viral RNA and effects of inhibitors of RNA synthesis. Virology **30:** 303–312.
16. **Sweet, B. H., and A. B. Sabin.** 1954. Properties and antigenic relationships of hemagglutinins associated with the dengue viruses. J. Immunol. **73:**363–373.
17. **Tarentino, A. L., and F. Maley.** 1974. Purification and properties of an endo-β-*N*-acetylglucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249:**811–817.
18. **Zhao, B., E. Mackow, A. Buckler-White, L. Markoff, R. M. Chanock, C.-J. Lai, and Y. Makino.** 1986. Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology **155:**77–88.