Acta virol. **33** : 497—503, 1989

EXPRESSION OF ENVELOPE GLYCOPROTEIN (E) OF JAPANESE ENCEPHALITIS VIRUS BY RECOMBINANT VACCINIA VIRUS

SHOZO HAISHI, HIDEKI IMAI*, KANJI HIRAI**, AKIRA IGARASHI, SHIRO KATO***

Department of Virology, Institute of Tropical Medicine, Nagasaki University, 12-4 Sakamoto-machi, Nagasaki City, 852 Japan, *Bioactive Science Institute, Nihon Zohki, Co. Ltd., Kinashigawa Kitayama, Yashiro-machi, Kato-gun, Hyogo Prefecture, 673-14 Japan, Department of Molecular Biology-1, Tokai University School of Medicine, Boseidai, Isehara City, 259-11 Japan, ***Department of Infectious Pathology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita City 565 Japan

Received June 6, 1988

Summary. — Vaccinia virus recombinants inserted with cDNA clones of Japanese encephalitis (JE) virus envelope glycoprotein (E) gene were constructed. The E gene product was detected in the recombinant virus-infected BHK21 cells by immunofluorescence (IF) and Western blotting. The intensity of IF observed was higher by the recombinant of the TK promoter — P7.5 promoter — inserted cDNA construct than by the P7.5 promoter — TK promoter — inserted cDNA construct. The E gene product was hardly detected by the recombinant carrying the TK promoter only upstream to the inserted cDNA, although the glycoprotein E mRNA had been transcribed.

Key words: *Japanese encephalitis virus*; *vaccinia virus*; *recombinant DNA expression*; *envelope gylcoprotein*

Introduction

Japanese encephalitis (JE) is a serious public health problem in several developing countries in Asia, with high mortality and grave sequelae (Umena *et al.*, 1985). Formalin-inactivated and highly purified JE vaccine from infected mouse brains was developed in Japan with proven safety and efficacy (Hammon *et al.*, 1971). Similar JE vaccine was produced in the Republic of Korea, and recently also in India and Thailand, and China has developed its own inactivated JE vaccine from infected hamster kidney cell cultures (Huang, 1982). However, the cost and supply of the current vaccines appear to limit mass-vaccination to control JE in presently epidemic area, and development of the second generation JE vaccine by recombinant DNA technology was recommended by the World Health Organization.

Like other flaviviruses, JE virus has 3 structural proteins (Shapiro *et al.*, 1979): the membrane protein (V1 or M), a core protein (V2 or C), and an

envelope glycoprotein (V3 or E). The E protein carries the virus neutralizing and protective epitope (Kimura-Kuroda and Yasui, 1983, 1986). The JE virus genome RNA consists of 10,976 nucleotides with essentially similar structure to other flavivirus genomes (Rice *et al.*, 1985; Castle *et al.*, 1985; Wengler *et al.*, 1985; Zhao *et al.*, 1986). Structural protein genes were located from the 5'terminus of a long open reading frame in the order of C, PreM (precursor of M), and E, followed by nonstructural protein genes (Sumiyoshi *et al.*, 1986, 1987). In order to develop the second generation JE vaccine, we constructed recombinant vaccinia viruses carrying the E gene cDNA and demonstrated expression of the E gene in infected cells.

Materials and Methods

Virus and cell culture. Vaccinia virus (VV), LC16m8 strain (Sugimoto *et al.*, 1985), was obtained from Prof. S. Hashizume, Chiba University. The virus was plaque-purified twice and grown in RK-13 cells at 37 °C for 3 days. The infected cells were sonicated and cytoplasmic extract was used as seed virus. A wild strain, JaOArS982, of JE virus was originally supplied by Dr. N. Ueba, Osaka Prefectural Institute of Public Health. RK-13 and BHK21 cells were grown in Eagle's minimal essential medium (MEM, Eagle, 1959) with 10% foetal calf serum (FCS). Thymidine kinaseless (TK^-) Fischer rat cell line, F2408, was kindly supplied by Prof. A. Hakura, Department of Oncogenic Virology, Research Institute for Microbial Diseases, Osaka University, and was grown in Dulbecco's modification of MEM (Dulbecco, 1959) with 10% foetal calf serum (FCS).

Construction of VV recombinants. DNA fragment of 1.770 base pairs (from 929 to 2,649 nucleotide), which covers entire coding region of the E protein (from 978 to 2,477 nucleotide), was prepared by *Bgl*II and *Mlu*I digestion of a cDNA clone, S22, of JE virus RNA (Sumiyoshi *et al.*, 1986). The resulting DNA fragment was blunt-ended, ligated with 3 different *BamH*I linkers in order to adjust the reading frame, and inserted into the *BamH*I site of insertion vector (PZ2) downstream to the TK gene promoter (Weir *et al.*, 1982; Bajszar *et al.*, 1983). Calcium phosphate precipitated (Nakano *et al.*, 1982) chimeric insertion vector (PZ3E, Fig. 1a) was inoculated to RK13 cells which had been infected with VV, and recombinant VV was produced by homologous recombination. Cytoplasmic extract of the infected cells was inoculated to Fischer Rat cells in the medium containing 25 μg/ml of 5-bromodeoxyuridine, and recombinant VV were twice plaque-purified. In order to increase expression efficiency, another 2 insertion vectors (PZ3 and PZ4) with P7.5 promoter (Venkatesan *et al.*, 1981) were constructed from PZ3 in the following gene order: TK gene promoter — P7.5 promoter — E gene cDNA insert (Fig. 1b), while another (PZ4E) had the order of P7.5 promoter — TK gene promoter — E gene cDNA insert (Fig. 1c). Recombinant VV were prepared from these chimeric insertion vectors as described above.

Detection of the E gene expression. Recombinant VV were inoculated to BHK21 cells, and the E gene expression was examined at transcriptional level by blot-hybridization analysis of RNA (Huang and Wertz, 1982), and at translational level by Western blotting (Burnette, 1981) and indirect immunofluorescence (IF) using anti-JE polyclonal mouse serum. In blot-hybridization, RNA was extracted from the cells by guanidine isothiocyanate, separated from DNA by cesium chloride centrifugation, electrophoresed in formalin-agarose gel, transferred to nitrocellulose membrane and detected by a radiolabelled DNA probe. The probe was prepared from the same DNA fragment inserted to chimeric vectors and labelled by nick-translation using α-^{32}P-ATP (Amersham, England). In Western blotting, the cells were solubilized by boiling for 3 min with 1% SDS and 2.5% 2-mercaptoethanol; the polypeptides were separated by discontinuous polyacrylamide gel electrophoresis with 10% separation gel (Laemmli, 1970) and electrophoretically transferred to a nitrocellulose membrane. The membrane was inactivated with 3% casein in PBS, reacted with anti-JE polyclonal mouse serum (1 : 1000 dilution), and horseradish peroxidase-conjugated anti-mouse IgG from the goat (Cappel, U.S.A., at 1 : 1000 dilution). The immunoreactive polypeptides were visualized by 4-chloro-1-naphthol and hydro-

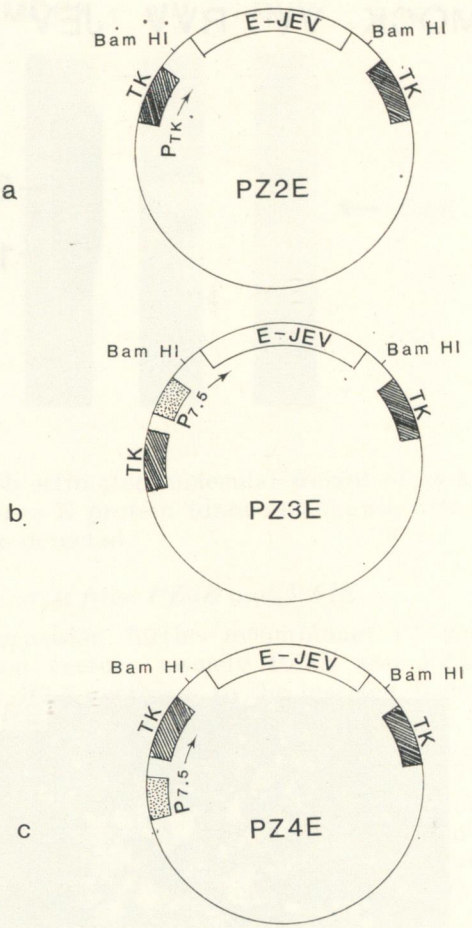

Fig. 1.

Construction of chimeric insertion vectors; (a) PZ2E, (b) PZ3E, and (c) PZ4E
E-JEV: E gene cDNA of JE virus
Direction of transcription is shown by an arrow.

gene peroxide. Fluorescent isothiocyanate-conjugated anti-mouse IgG from the rabbit (Cappel, U.S.A.) was used at 1 : 50 dilution for immunofluorescence staining. Anti-JE polyclonal mouse serum was prepared by repeated intraperitoneal inoculation of purified JE virus grown in cell culture.

Results

Expression of the E gene by a recombinant from PZ2E

In blot-hybridization analysis, RNA from BHK21 cells infected with a PZ2E recombinant VV showed weak but definite reaction, while the specimens from wild type VV-infected or mock-infected cells did not show any positive reaction. In contrast, RNA from JE virus-infected cells strongly reacted with the probe (Fig. 2). In the recombinant VV-infected cells, West-

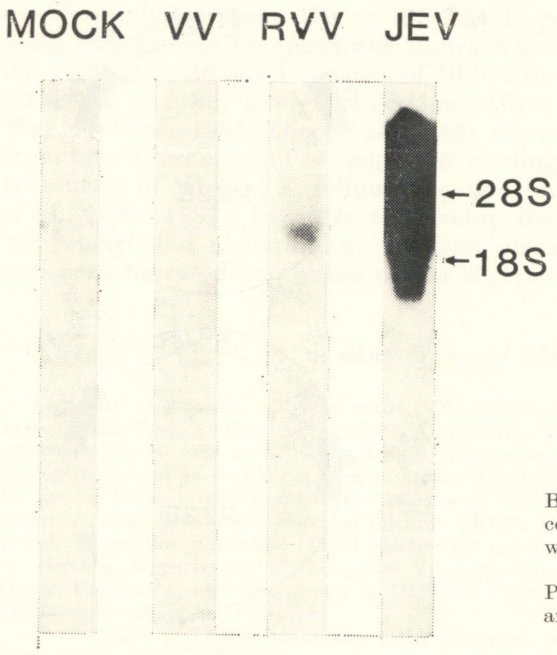

Fig. 2.
Blot hybridization of RNA from BHK21 cells mock-infected (MOCK), or infected with wild type VV (VV), recombinant VV (RVV), and JE virus (JEV) Position of 28S and 18S ribosomal RNA are shown by arrows.

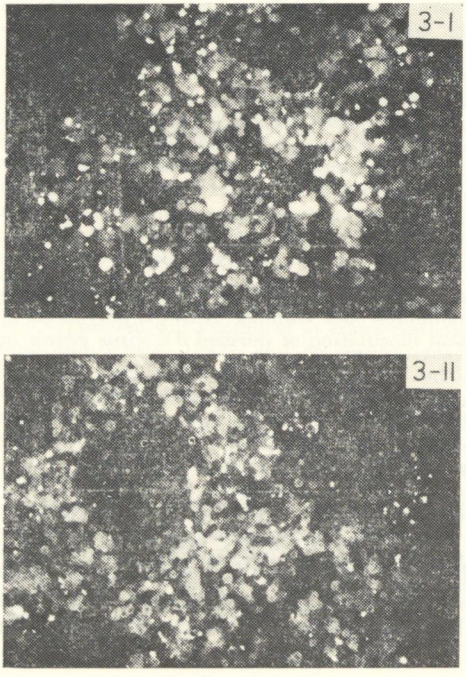

Fig. 3.
Immunofluorescence of BHK21 cells infected with recombinant VV from PZ3E (I) or PZ4E (II)

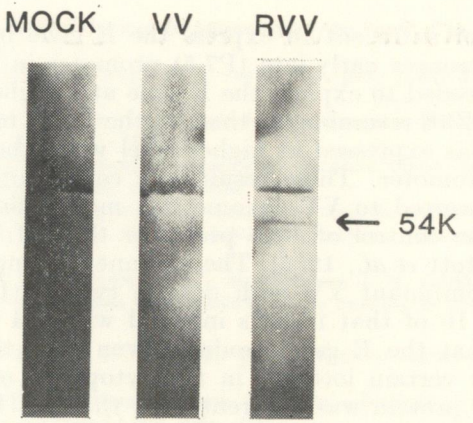

Fig. 4.
Western blotting on BHK21 cells mock-infected (MOCK), or infected with wild type VV (VV) and recombinant VV from PZ3E (RVV)
Position of 54 kD M_r protein is shown by an arrow.

ern blotting showed a faint band with estimated molecular weight of 54 kD corresponding to the value of JE virus E protein (data not shown). However, no specific fluorescence could be detected.

Expression of the E gene by recombinants from PZ3E and PZ4E

In order to increase the E gene expression, further recombinant VV were prepared from the chimeric insertion vectors, namely PZ3E and PZ4E, which carried the stronger promoter P7.5 upstream to the inserted E gene cDNA. BHK21 cells infected with these recombinants showed definite IF which was localized in the cytoplasm, and fluorescence intensity was stronger by PZ3E recombinant (Fig. 3-I) than by PZ4E recombinant (Fig. 3-II). The result indicated higher level of the E gene production by the PZ3E recombinant. Western blotting of BHK21 cells infected with the PZ3E recombinant showed an immunoreactive protein band with estimated molecular weight of 54 kD, which was not observed with mock-infected or wild type VV-infected cells (Fig. 4). Another band with molecular weight of 67 kD was observed in all specimens, probably showing nonspecific reaction to bovine serum albumin.

Discussion

Vaccinia virus (VV) had proved its safety and potential effectiveness as live vaccine through the smallpox eradication project. It has been recently used as an eukaryotic expression vector of foreign genes (Panicalli and Paoletti, 1982; Mackett *et al.*, 1982; Smith *et al.*, 1983; Mackett and Smith, 1986). Control of JE in a presently epidemic area requires a great amount of inexpensive vaccine, and a recombinant VV which could express JE virus E protein would be a suitable candidate. Present result is the first positive step aiming at this goal, although further studies on the immunogenicities of the recombinants and better conditions for expressing the E gene are required. Our results indicated that early gene (TK) promoter was

not sufficient to express the E gene in an amount detectable by IF, and stronger early-late (P7.5) promoter in conjunction with TK promoter was needed to express the E gene at a higher level. Stronger fluorescence by the PZ3E recombinant than by the PZ4E recombinant indicated that the E gene was expressed at higher level when the inserted E gene was closer to P7.5 promoter. These results are consistent with the reports that foreign gene inserted to VV genome was more efficiently expressed when it was under the control of P7.5 promoter than of TK promoter (Cochran *et al.*, 1985; Stott *et al.*, 1986). The amount of dengue virus proteins produced by a recombinant VV with dengue type 4 (D4) virus cDNA was approximately 1/10 of that in cells infected with D4 virus (Zhao *et al.*, 1987). IF showed that the E gene product, even with the PZ3E recombinant, accumulated at certain location in the cytoplasm of infected cells. This distribution of E protein was different from that in JE virus-infected cells, in which fluorescence was distributed in whole cytoplasm with higher intensity in perinuclear region. This difference may be due to different replication strategies of JE virus and VV, or may indicate that other JE virus gene(s) or gene product(s) are required for the proper transport of the expressed E protein.

Acknowledgements. This work was supported by the Grant in Aid for Developmental Research from the Ministry of Education, Science and Culture of Japan, No. 61870025, and by the Research Grant from the World Health Organization, Regional Office for the Western Pacific. Valuable suggestions for the construction of VV recombinants by Dr. B. Moss were greatly appreciated.

References

Bajszar, G., Wittek, R., Weir, J., and Moss, B. (1983): Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. *J. Virol.* **46**, 530–537.

Burnette, W. N. (1981): "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195–203.

Castle, E., Nowak, T., Leidner, U., Wengler, G., and Wengler, G. (1985): Sequence analysis of the viral core protein and the membrane-associated proteins V1 and NV1 of the West Nile virus and the genome sequence for these proteins. *Virology* **145**, 227–236.

Cochran, M. A., Puckett, C., and Moss, B. (1985): *In vitro* mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. *J. Virol.* **54**, 30–37.

Dulbecco, R., and Freeman, G. (1959): Plaque production by the polyoma virus. *Virology* **8**, 396–397.

Eagle, H. (1959): Amino acid metabolism in mammalian cell cultures. *Science* **130**, 432–437.

Hammon, W. McD., Kitaoka, M., and Downs, W. G. (1971): Immunization for Japanese Encephalitis. Igaku-shoin, Tokyo.

Huang, C. H. (1982): Studies on Japanese encephalitis in China. *Adv. Virus Res.* **27**, 71–101.

Huang, Y. T., and Wertz, G. W. (1982): The genome of respiratory syncytial virus as a negative-stranded RNA that codes for at least seven mRNA species. *J. Virol.* **43**, 150–157.

Kumura-Kuroda, J., and Yasui, K. (1983): Topographical analysis of antigenic determinants on envelope glycoprotein V3(E) of Japanese encephalitis virus, using monoclonal antibodies. *J. Virol.* **45**, 124–132.

Kumura-Kuroda, J., and Yasui, K. (1986): Antigenic comparison of envelope protein E between Japanese encephalitis virus and some other flaviviruses using monoclonal antibodies. *J. gen. Virol.* **67**, 2663–2672.

Laemmli, U. K. (1970): Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**, 680–685.

Mackett, M., and Smith, G. L. (1986): Vaccinia virus expression vectors. *J. gen. Virol.* **67**, 2067–2082.

Mackett, M., Smith, G. L., and Moss, B. (1982): Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proc. natn. Acad. Sci.* **79**, 7415–7419.

Nakano, E., Panicalli, D., and Pailetti, E. (1982): Molecular genetics of vaccinia virus: demonstration of marker rescue. *Proc. natn. Acad. Sci.* **79**, 1593–1596.

Panicalli, D., and Paoletti, E. (1982): Construction of poxvirus as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proc. natn. Acad. Sci.* **79**, 4927–4931.

Rice. C. M., Lenches, E. M., Eddy, S. R., Shin, S.-J., Sheets, R. L., and Strauss, J. H. (1985): Nucleotide sequence of yellow fever virus: implication for flavivirus gene expression and evolution. *Science* **229**, 726–733.

Shapiro, D., Brandt, W. E., Cardiff, R. D., and Russell, P. K. (1979): The proteins of Japanese encephalitis virus. *Virology* **44**, 108–124.

Smith, G. L., Mackett, M., and Moss, B. (1983): Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. *Nature (Lond.)* **302**, 490–495.

Stott, E. J., Ball, L. A., Young, K. K., Furze, J., and Wertz, G. W. (1986): Human respiratory syncytial virus glycoprotein G expressed from a recombinant vaccinia virus vector protects mice against live-virus challenge. *J. Virol.* **60**, 607–613.

Sugimoto, M., Yasuda, A., Miki, K., Morita, M., Suzuki, K., Uchida, N., and Hashizume, S. (1985): Gene structures of low-neurovirulent vaccinia virus LC16mO, and their Lister original (LO) strains. *Microbiol. Immunol.* **29**, 421–428.

Sumiyoshi, H., Morita, K., Mori, C., Fuke, I., Shiba, T., Sakaki, Y., and Igarashi, A. (1986): Sequence of 3000 nucleotides at the 5′ end of Japanese encephalitis virus RNA. *Gene* **48**, 195–201.

Sumiyoshi, H., Mori, C., Fuke, I., Morita, K., Kuhara, S., Kondou, I., Kukuchi, Y., Nasgamatsu, H., and Igarashi, A. (1887): Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* **161**, 497–510.

Umenai, T., Krzysko, R., Bektimorov, A., and Assaad, F. A. (1985): Japanese encephalitis: current worldwide status. *Bull. World Hlth. Org.* **63**, 625–631.

Venkatesan, S., Baroudy, B. M., and Moss, B. (1981): Distinctive nucleotide sequence adjacent to multiple initiation and termination sites of an early vaccinia virus gene. *Cell* **125**, 805–813.

Weir, J. P., Bajszar, G., and Moss, B. (1982): Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA. *Proc. natn. Acad. Sci.* **79**, 1210–1214.

Wemgler, G., Castle, E., Leidner, U., Nowak, T., and Wengler, G. (1985): Sequence analysis of the membrane protein V3 of the flavivirus West Nile virus and its gene. *Virology* **147**, 264–274.

Zhao, B., Mackow, E., Buckler-White, A., Markoff, L., Chanock, R. M., Lai, C.-J., and Makino, Y. (1986): Cloning full length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. *Virology* **155**, 77–88.

Zhao, B., Prince, G., Horswood, R., Eckels, K., Summers, P., Chanock, R., and Lai, C.-J. (1987): Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. *J. Virol.* **61**, 4019–4022.