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Infectious Japanese Encephalitis Virus RNA Can Be Synthesized from In Vitro-Ligated cDNA Templates

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Japanese encephalitis virus (JEV) is a positive-stranded enveloped RNA virus that belongs to the family *Flaviviridae*. Genomic JEV RNA is approximately 11 kb long and encodes 10 proteins, 3 structural and 7 nonstructural. A full-length cDNA copy of the JEV genome was constructed by in vitro ligation of two cDNA fragments which encode the 5′ (nucleotide positions 1 to 5576) and 3′ (nucleotide positions 5577 to 10976) halves of the genome. T7 RNA polymerase transcripts of the ligated full-length cDNA template were infectious when transfected into BHK-21 cells. To identify the recombinant virus, a silent mutation was introduced into the clone encoding the 3′ half of the genome, which abolished an *XbaI* site at nucleotide position 9131. Virus recovered by transfection with the transcripts contained this silent mutation, confirming its identity. Recombinant and parent viruses were identical with respect to growth and plaque production in BHK-21 cells, envelope protein expression in C6/36 cells, and neurovirulence and immunogenicity in mice. Repeated attempts to obtain infectious RNA by transcription from full-length JEV genome cDNA templates cloned into plasmid vectors were unsuccessful. Synthesis of infectious JEV RNA from in vitro-ligated JEV cDNA templates will be useful for molecular and genetic studies of flavivirus replication and virulence.

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus, found primarily in southeast Asia and the Pacific archipelagos, that causes severe encephalitic and neurologic disease manifestations (4). During epidemic periods, fatality rates as high as 20% have been noted, particularly among the immunologically naive and those at the extremes of the age spectrum. Hoke et al. (14) reported that the incidence of JEV has approached 16% during some epidemics in Asia, which approaches the incidence observed during poliomyelitis outbreaks in the United States during the 1950s. For these reasons, the development of safe and potent JEV vaccines for human use and understanding of the molecular genetics of virulence are World Health Organization vaccine development priorities (3).

Sequence analysis of the genomic RNAs of several flaviviruses has revealed that they are organized similarly (31). Genomes of the virulent JEV strains JaOArS982 (33), Nakayama (23), Beijing (13), and SA-14 (24) and of the attenuated vaccine strain SA-1414-2 (24) have been cloned and sequenced, and the genomes have been analyzed at the molecular level. On the basis of nucleotide sequence analysis, JEV strains can be divided into three different groups representing viruses from northern Thailand and Cambodia; southern Thailand, Malaysia, and Indonesia; and Japan, China, Taiwan, the Philippines, and the Indian subcontinent (6). Antigenic variation has been reported by using both polyclonal (12, 25, 26) and monoclonal antibodies (MAb) to the E protein as assayed by reactivity in the enzyme-linked immunosorbent assay and in complement fixation and neutralization tests (16, 17).

The JEV genome is a single-stranded RNA molecule, approximately 11 kb in length, of positive sense coding for three structural proteins designated capsid (C), membrane (M), and envelope (E) and seven nonstructural proteins designated NS1 through NS5 (13, 24, 33). The genome is translated into a polyprotein from which structural and nonstructural proteins are processed by cellular (1, 32) and viral proteases (5, 28, 36). The biological functions and antigenic structure of the flavivirus E protein have been extensively studied; however, the processes of virion morphogenesis, encapsidation of the genomic RNA with C protein, and biological activities of the nonstructural proteins remain largely unknown. Nonstructural protein NS3 is a virus-encoded trypsinlike serine proteinase involved in cleavage of NS2B, NS3, NS4A, and probably NS5 during morphogenesis in the rough endoplasmic reticulum (7, 28, 36). Subcellular fractions rich in flavivirus RNA polymerase activity are enriched in NS5 and NS3, which are thought to be components of the RNA polymerase (7, 10, 37). Although biochemical analyses of flavivirus protein function have elucidated some features of their biological importance, the relationship of these functions to virus virulence and disease is poorly understood.

Detailed sequence comparisons between the virulent and attenuated flaviviruses yellow fever virus (YFV) (11), dengue virus (2), and JEV (24) have implicated specific genomic regions as major determinants of virulence; however, there are numerous nucleotide differences in the sequences which are obviously unrelated to biological function (2, 11, 20, 21, 24). Assignment of particular mutations to specific phenotypes has been possible through construction of full-length cDNA clones from which infectious progeny virus can be produced (8, 15, 19, 27, 30). The availability of complete copies of the genome in the form of cDNA allows construction of strain pairs that are isogeneic except for defined substitutions. With availability of the JaOArS982 infectious clone, it is now possible to study the effects of specific defined nucleotide changes on biological function of the 3′ and 5′ noncoding regions, mechanism of RNA synthesis, polyprotein processing, and virulence of JEV virus.

Many attempts have been made to develop full-length

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flavivirus cDNA clones from which infectious RNA can be transcribed. The first successful system for transcription of infectious flavivirus RNA from a cDNA template was developed for YFV by Rice et al. (29). These workers ligated three subgenomic cDNA fragments in vitro to form full-length template from which infectious RNA could be transcribed. Recently, Lai et al. (18) reported development of a full-length dengue type 4 virus cDNA clone from which infectious RNA can be transcribed.

In this study, we report transcription of infectious JEV RNA from a full-length JEV cDNA template constructed by in vitro ligation of two subgenomic JEV cDNA clones. Although several full-length JEV cDNA clones were constructed in the bacterial plasmid pBR322 in *Escherichia coli* HB101, infectious JEV RNA could not be transcribed from them because of mutations which occurred in the full-length clones during replication in the bacterial host.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS)–2 mM L-glutamine–0.1 mM MEM nonessential amino acids–0.3% sodium bicarbonate–10 U of penicillin per ml–10 μg of streptomycin per ml. Wild-type JEV strain JaOArS982 was grown in C6/36 cells and purified, and genomic RNA was extracted as previously described (33).

Plaque assay. Monolayers of BHK-21 cells in 35- or 60-mm-diameter dishes were used for plaque assays. After virus inoculation, the cells were washed with phosphate-buffered saline (PBS) and overlaid with 1% agarose containing 2% FCS in MEM. Cells were cultured at 37°C for 3 to 5 days and stained with neutral red or crystal violet to visualize plaques.

Construction of cDNA clones. DNA manipulations were done as previously described (33). For cDNA synthesis, 3′-end and 5′-end oligonucleotide primers were prepared whose sequence is based on the nucleotide sequence of JEV strain JaOArS982 (33). The 3′-end primer contained a *Kpn*I site and 25 nucleotides of the negative-sense 3′-terminal region of the JEV genomic RNA. The 5′-end primer contained a *Sal*I site, a T7 RNA polymerase promoter sequence, and 25 nucleotides of the positive-sense 5′-terminal region of the JEV genomic RNA. Genomic RNA and the 3′-end primer were used for first-strand synthesis, using reverse transcriptase (Seikagaku America, Inc., Rockville, Md.). First-strand cDNA and the 5′-end primer were used for second-strand cDNA synthesis, using T4 DNA polymerase and T4 gene 32 product. After synthesis, the cDNA was cut with *Bam*HI and *Sal*I, and the digested cDNAs were inserted into the *Bam*HI and *Sal*I sites of pBR322 and used to transform competent *E. coli* HB101. JEV cDNA clones pM343 and pH756 were obtained from this cDNA library. The *Pvu*II-*Sal*I fragment, which begins at nucleotide 8782 and extends to the 3′ end of the genomic cDNA, was cut from the 3′-half clone pH756 and subcloned into pUC119 (35). This clone was modified by oligonucleotide site-directed mutagenesis (Amersham Corp., Arlington, Heights, Ill.) to remove the *Xba*I site at nucleotide 9131 and to introduce an *Xba*I site at the end of the JEV sequence. The *Sst*II-*Sal*I fragment, extending from nucleotide 8967 to the 3′ end of the genomic cDNA, in clone pH756 was replaced with the mutagenized fragment to generate cDNA clone pH756X.

Construction of full-length cDNA template for in vitro RNA transcription. To construct by in vitro ligation the full-length DNA copy of JEV genomic RNA designated IC37, the 5′-half clone pM343 which contained the T7 RNA promoter (Fig. 1A and 2A) and JEV sequence from positions 1 to 5576 was digested with *Sal*I and *Bam*HI. The DNA fragment M343 was purified by gel electrophoresis and quantitated, and its purity was verified by gel electrophoresis. To prepare cDNA containing the sequence of the 3′ half of the JEV genome, pH756 DNA was digested with *Bam*HI and *Kpn*I (Fig. 1A); the cDNA fragment of 5,400 bp which contained cDNA from JEV position 5577 to the end of the genome with an additional C residue (Fig. 2B) was purified by agarose gel electrophoresis and quantitated, and its purity was verified by agarose gel electrophoresis. To ligate the 3′ and 5′ clones together, 3 μg of purified M343 fragment and 10 μg of H756 DNA fragment were placed in a 100-μl ligation reaction overnight at 4°C. The ligation products were digested with *Kpn*I for 60 min at 37°C; then they were digested with proteinase K (100 μg/ml)–0.2% sodium dodecyl sulfate for 30 min at 37°C. The mixture was then extracted twice with phenol-chloroform and precipitated with ethanol.

A full-length cDNA template of the JEV genome which would transcribe into RNA having the actual terminal sequence of JEV genomic RNA was also prepared. The 3′ cDNA clone H756X was cut with *Bam*HI and *Xba*I, and the DNA fragment (nucleotide positions 5577 to 10976 plus an additional four-nucleotide GATC sequence; Fig. 2C) was purified by agarose gel electrophoresis. The fragment designated H765X was ligated to purified M343 DNA as before, and the ligation products were digested with *Xba*I to facilitate synthesis of full-length viral RNA and treated with mung bean nuclease to remove the four extra bases which are part of the *Xba*I restriction site. The full-length cDNA was then used as a template for JEV RNA transcription.

In vitro RNA transcription. T7 RNA polymerase was purified from *E. coli* BL21 transformed with clone pAR1219 which contained the T7 RNA polymerase gene (34). The RNA polymerase was purified from the cells as described previously (9). For RNA synthesis, 0.2 μl of the T7 RNA polymerase preparation was added to a 50-μl reaction mixture which contained 1 μg of ligated template DNA, 40 mM Tris (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 1 mM each CTP, UTP, and GTP, 0.1 mM ATP, 1 mM $m^7G(5')ppp(5')A$, and 5 U of RNase inhibitor. The reaction mixture was incubated for 30 min at 37°C.

RNA transfection. Subconfluent monolayers of BHK-21 cells in 35-mm-diameter, 6-well tissue culture plates were used for RNA transfection. The cells were washed with PBS and incubated at room temperature for 10 min with 400 μl of PBS containing either RNA transcripts or genomic RNA and 50 μl of lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.). The cells were then washed with PBS and overlaid with MEM containing 1% agarose and 2% FCS. After being in culture for 3 to 5 days, the cells were stained with neutral red to visualize the virus plaques.

Immunofluorescence assay. The immunofluorescence assay was performed by the method of Lyerla and Forrester (22). C6/36 cells infected with parent or recombinant viruses were reacted with MAb J93 mAF T1 specific for the JEV envelope (E) protein (supplied by E. Henchal). Fluorescein-conjugated goat anti-mouse immunoglobulin was used to localize JEV E protein expression in acetone-fixed cells.

Virulence and neutralizing antibody in mice. Groups of eight 3-week-old Swiss ICR male mice were used for virus virulence assays. Mice were inoculated by the intraperitoneal route with 100 PFU of parent or recombinant IC37 virus after intracerebral inoculation with 20 μl of PBS (IP/IC

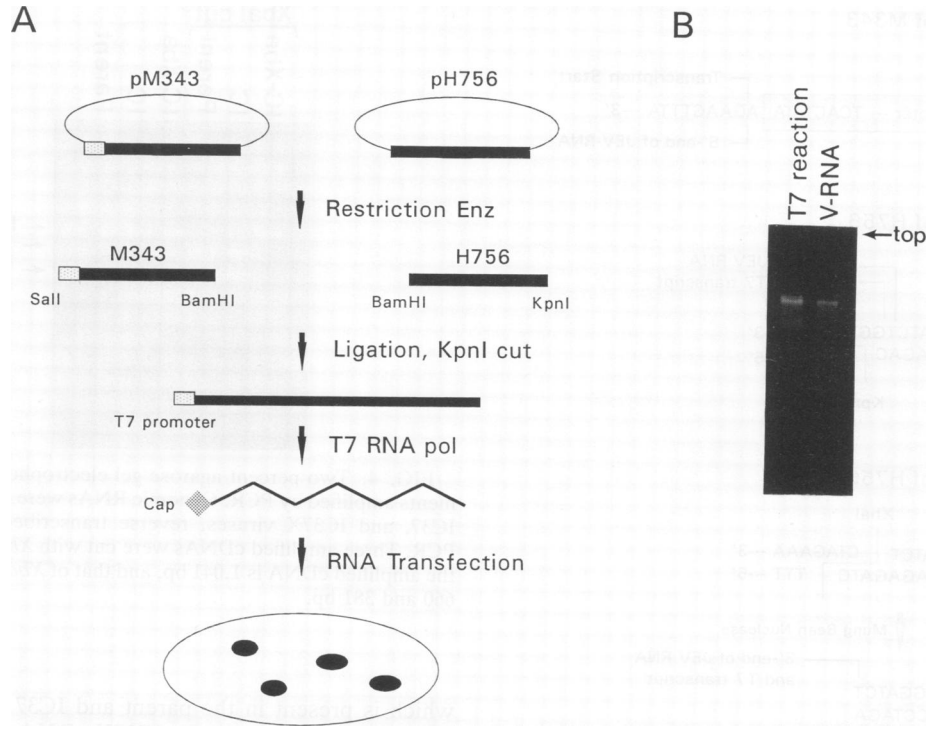

FIG. 1. Construction of cDNA template. (A) Strategy for recovering JEV from cDNA. JEV cDNA fragment M343, which has the T7 RNA promoter and JEV sequence from nucleotides 1 to 5576, was prepared from JEV cDNA clone pM343 by cutting *Sal*I and *Bam*HI. Fragment H756, which has the JEV sequence from nucleotides 5577 to 10976 and an additional C residue, was prepared from JEV cDNA clone pH756 by digestion with *Bam*HI and *Kpn*I. These two fragments were ligated, digested with *Kpn*I, treated with proteinase K, extracted with phenol-chloroform, precipitated with ethanol, and used for T7 RNA polymerase reaction as the full-length JEV cDNA template IC37. Transcription products were transfected into BHK-21 cells with lipofectin. (B) Formaldehyde-denatured 0.8% agarose electrophoresis of viral genomic RNA and the T7 RNA transcription products.

inoculation). Fourteen days after inoculation, serum was collected from mice which survived IP/IC challenge with 100 PFU of recombinant or parent virus and analyzed for serum neutralizing antibody. Titers were determined by plaque reduction neutralization as follows. Fifty microliters of 40-, 80-, 160-, 320-, 640-, and 1,280-fold diluted serum was incubated with 50 PFU of parent virus at 4°C overnight. After incubation, BHK-21 monolayers were inoculated with the virus-antibody mixture, overlaid for plaque formation, cultured for 3 to 5 days, and stained with neutral red to visualize plaques. The serum dilution which reduced the plaque count by 70% was recorded as the neutralizing antibody titer. Fifteen days after primary infection with parent or IC37 virus, surviving mice were challenged by the IP/IC route of inoculation with 100 PFU of parent virus and observed for signs of illness.

RESULTS

In vitro synthesis of infectious RNA. Full-length cDNA templates, for in vitro RNA synthesis of JEV RNA, were prepared by ligation of cloned cDNAs containing the 5′ (M343) and 3′ (H756 or H756X) halves of the genome (Materials and Methods; Fig. 1A). Full-length virus cDNA, derived from ligation of subgenomic clones M343 and H756 to produce clone IC37, was cut with *Kpn*I to obtain a template for RNA runoff products (Fig. 2B). The cDNA derived from ligation of clone M343 and H756X to produce clone IC37X was cut with *Xba*I and treated with mung bean nuclease to obtain a template for RNA runoff products (Fig. 2C). RNA transcripts derived from IC37 were examined by electrophoresis on formaldehyde-denatured 0.8% agarose gels (Fig. 1B). Full-length transcripts and viral genomic RNA migrated at the same position. Half-length transcripts observed on the gels were thought to be transcribed from the unligated 5′-half cDNA fragment M343 which contained the T7 promoter (Fig. 1A and 2A). The amount of half-sized RNA transcripts obtained in synthesis reactions was reduced by increasing the molar ratio of the 3′-half cDNA fragment H756 to three times more than that of the 5′-half cDNA fragment in the ligation reaction. Approximately 5 μg of full-length transcribed RNA was obtained from 0.2 μg of ligated cDNA template in a 50-μl T7 RNA polymerase reaction mixture.

As shown in Fig. 2A, T7 RNA polymerase was expected to initiate transcription at the 5′-terminal A residue of the JEV cDNA. Full-length runoff RNA products derived from IC37X should therefore have the same 5′ and 3′ ends as authentic JEV genomic RNA (Fig. 2C). Although RNA transcripts of IC37 contained an extra C residue at the 3′ end, this extra C did not affect the infectivity of the RNA transcripts. The infectivity of RNA synthesized from the ligated template was approximately 100 PFU per μg and that of genomic RNA was 10^5 PFU per μg. The reason for this discrepancy in infectivity is not known.

Characterization of recovered JEVs. RNA transcripts from ligated full-length cDNA templates IC37 and IC37X and viral genomic RNA were transfected with lipofectin onto BHK-21 cells. After 3 to 5 days in culture, cells were stained with neutral red to reveal virus plaques. No significant differences

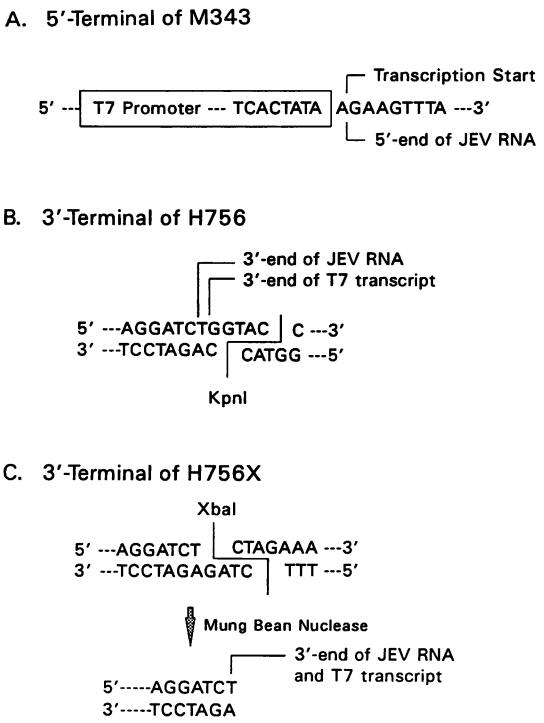

FIG. 2. 5' and 3' termini of cDNA clones. (A) The 5' terminus of JEV cDNA M343, which was used for construction of full-length cDNA templates for T7 RNA synthesis. (B) 3' terminus of JEV cDNA H756. The full-length cDNA template IC37 was derived from ligation of M343 and H756 and cut with *Kpn*I to produce T7 RNA polymerase runoff products. (C) 3' terminus of JEV cDNA H756X. IC37X was derived from ligation of cloned cDNAs of M343 and H756X, cut with *Xba*I, and treated with mung bean nuclease.

in the size or morphology of plaques were observed in the BHK-21 cell monolayers transfected with RNAs transcribed from the template cDNAs IC37 and IC37X or with purified RNA from parent virus or in cells infected with parent virus or recombinant viruses IC37 or IC37X. Plaques produced by the parent and recombinant IC37 viruses after 5 days of culture in BHK-21 cell culture are shown in Fig. 3.

To verify that the virus recovered from transfection was JEV derived from cloned cDNA, genomic RNAs were purified from the JEV parent virus and from the IC37 and IC37X recombinant viruses. These RNAs were examined for the presence of the *Xba*I restriction site at nucleotide 9131,

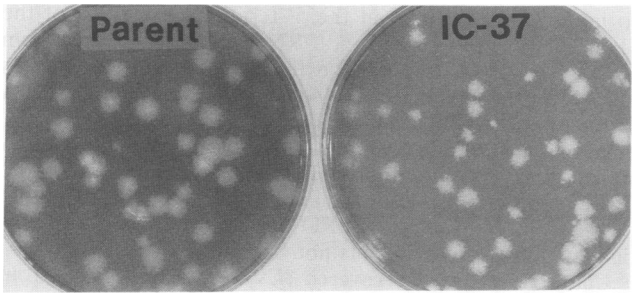

FIG. 3. Plaque morphology of parent JaOArS982 and IC37 viruses. Monolayers of BHK-21 cells were inoculated with parent or IC37 viruses, overlaid with agarose, and stained with crystal violet after 5 days in culture.

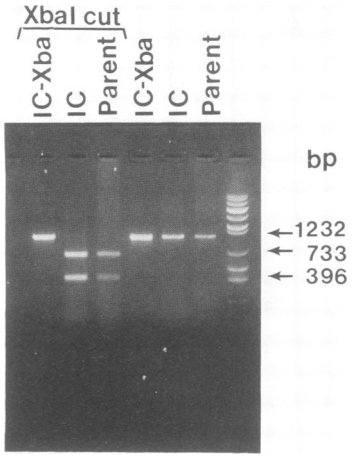

FIG. 4. Two percent agarose gel electrophoresis of cDNA fragments amplified by PCR. Genomic RNAs were purified from parent, IC37, and IC37X viruses, reverse transcribed, and amplified by PCR. Three amplified cDNAs were cut with *Xba*I. Expected size of the amplified cDNA is 1,041 bp, and that of *Xba*I-digested cDNAs is 660 and 381 bp.

which is present in the parent and IC37 virus cDNA. The *Xba*I restriction site in the IC37X cDNA had been removed by site-directed in vitro mutagenesis. A positive-sense 18-mer which would anneal to genomic RNA at nucleotide positions 8471 to 8488, a negative-sense 18-mer which annealed at JEV nucleotide positions 9494 to 9511, and purified viral genomic RNAs were used for cDNA synthesis and polymerase chain reaction (PCR) amplification. As shown in Fig. 4, cDNAs of 1,041 bp, representing the PCR-amplified fragment, were observed by agarose gel as expected. When these cDNAs were digested with *Xba*I, cDNAs from parent and recombinant IC37 viruses were cleaved into 660- and 381-bp fragments; however, cDNA from recombinant IC37X virus was not digested. These results confirmed the identity of recombinant IC37X viral genomic RNA, which retained the point mutation introduced into the cDNA.

Growth rates of parent, IC37, and IC37X viruses were compared in BHK-21 cells infected at multiplicities of 0.01 and 0.1 PFU per cell (Fig. 5). There were no significant differences in growth rates among viruses in BHK-21 cells infected at either multiplicity. The maximum virus yield for parent, recombinant IC37, and recombinant IC37X viruses in BHK-21 cells was about 10^8 PFU/ml after 4 days of incubation. The expression of JEV E protein in cells infected with parent or IC37 virus was examined by using J93 MAb, which reacts only with JEV E protein and with no other flavivirus antigen. The cytoplasm of cells infected with either parent or IC37 virus was uniformly stained with antibody (Fig. 6B and D), confirming the expression and antigenic identity of the viruses. Uninfected cells were not stained (Fig. 6F).

Neurovirulence and neutralizing antibody production in mice. To compare neurovirulence of parent and recombinant IC37 viruses, 3-week-old Swiss ICR male mice were inoculated with these JEVs by the IP/IC route. As shown in Fig. 7, seven of eight mice died after inoculation with parent virus. The average survival time was 8.9 days ± 0.8, with a range of 7 to 10 days. Six of eight mice died after inoculation with IC37 virus. The average survival time was 9.5 days ± 1.6 days, with a range of 7 to 11 days. The neurovirulence of

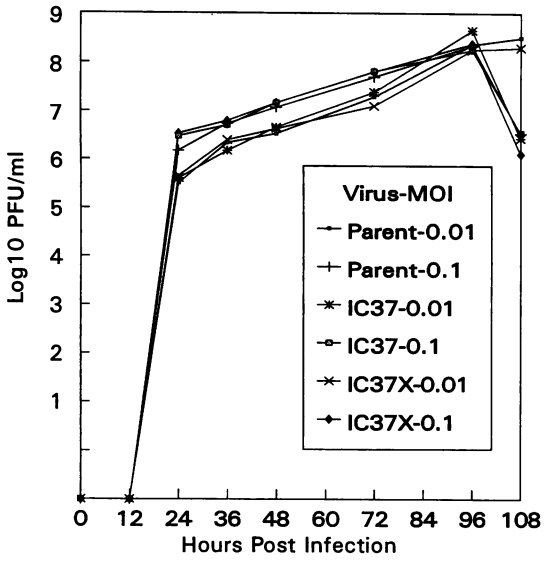

FIG. 5. JEV growth in BHK-21 cells. Cells were infected at multiplicities of 0.01 or 0.1 PFU per cell with parent or recombinant IC37 or IC37X virus. After the inoculation, the culture media were harvested at various intervals and virus titers were determined by plaque assay.

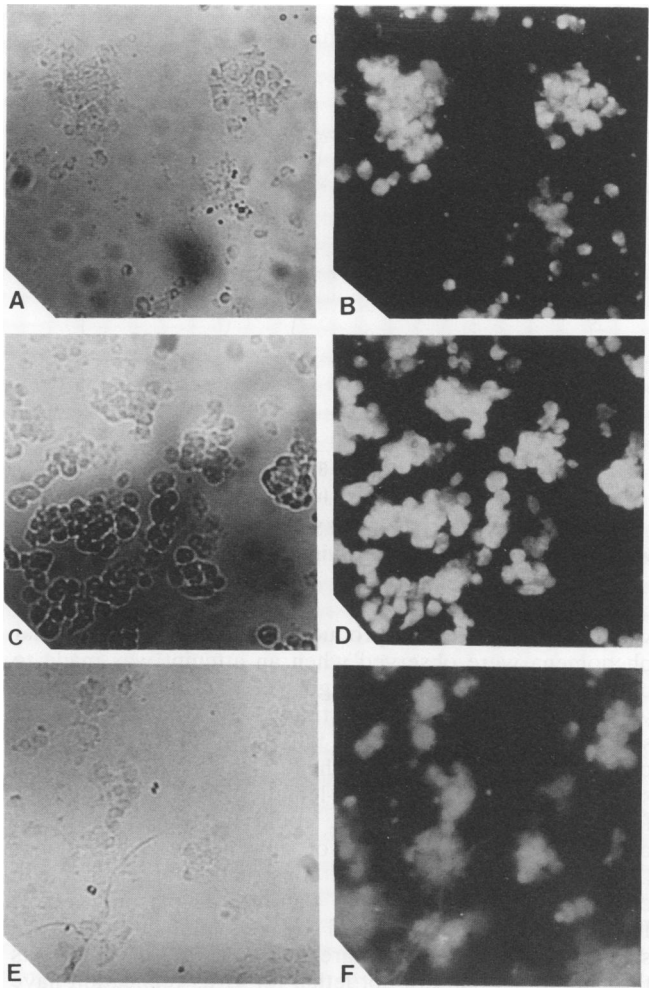

FIG. 6. JEV E protein expression in C6/36 cells, detected by indirect immunofluorescence. C6/36 cells mock infected (E and F) or infected with parent JaOArS982 virus (A and B) or IC37 virus (C and D) were cultured for 3 days and fixed with cold acetone. JEV E protein-specific mouse MAb and fluorescein-conjugated goat anti-mouse immunoglobulin were used to detect the JEV E protein expressed in the cells. Panels A and B, C and D, and E and F are photographs of the same field, panels A, C, and E by phase microscope and panels B, D, and F by fluorescence microscope.

parent JaOArS982 and IC37 viruses for 3-week-old mice was similar.

To compare neutralizing antibody responses of mice to the parent and recombinant IC37 viruses, serum was taken 14 days postinfection from mice that survived challenge with the parent or recombinant viruses. Plaque reduction neutralization titers of 160 or greater were obtained with serum of mice that survived primary challenge with parent virus. Titers of 80 and 160 were obtained with serum specimens from mice challenged with IC37 virus. Mice that survived challenge with parent or recombinant IC37 viruses were subsequently challenged by the IP/IC route with 100 PFU of parent virus. All mice survived the secondary IP/IC challenge with the virulent parent virus. These results indicated that mice that survived the first IP/IC challenge with parent or recombinant virus produced antibody that protected the animals from JEV encephalitis after the secondary IP/IC virus challenge.

DISCUSSION

Full-length JEV cDNAs were constructed by in vitro ligation of two cDNAs which contained the 5′ and 3′ halves of JEV genome cDNA. Infectious RNA transcripts of the virus genome were synthesized from the ligated full-length template with T7 RNA polymerase. The plaque size, growth rate in BHK-21 cells, neurovirulence for mice, and immunogenicity in mice of parent virus and recovered virus from transcripts were the same. C6/36 cells infected with recombinant IC37 and the parent virus reacted to JEV E protein-specific J93 MAb. Plaque reduction antibody titers of 80 to 160 were observed in the serum of surviving mice that received IC37 virus. Mice that survived infection with IC37 virus were also protected from secondary challenge with the virulent parent virus. Genomic RNA from virus recovered by transcription of ligated cDNA template contained the point mutation introduced into the template cDNA. We therefore concluded that the recombinant IC37 virus recovered by transfection with RNA transcripts of virus cDNA was JEV.

We have tried several different approaches to construct full-length JEV cDNA clones in plasmids to use as templates for transcription of infectious RNA. Transcripts from more than 100 independent full-length cDNA clones were not infectious (data not shown). One of these full-length clones was sequenced, revealing a mutation in the E protein gene at nucleotide position 2175, which introduced an opal stop codon into the sequence. The cDNA of this region was replaced, and the sequence of the cDNA was confirmed. However, after replication of the plasmid in *E. coli*, RNA transcripts from the full-length repaired clone were not infectious. It appears that mutations were introduced into other regions of the full-length JEV cDNA during replication of the plasmid in *E. coli*. Perhaps the plasmid containing full-length JEV cDNA is toxic for *E. coli*, resulting in the introduction of mutations in the viral cDNA that are not

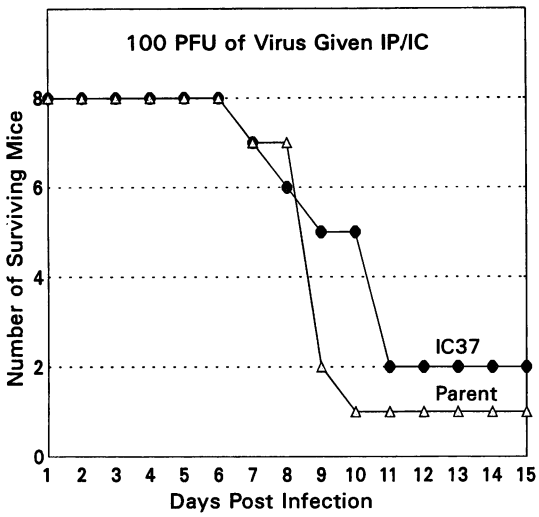

FIG. 7. Survival of 3-week-old Swiss ICR male mice challenged with 100 PFU of parent or IC37 virus by the IP/IC route.

viable as templates to produce infectious RNA. Similar phenomena were observed when an attempt was made to obtain transcripts of full-length YFV cDNA in plasmid vectors (29). Although infectious RNA transcripts were successfully obtained from a full-length YFV cDNA copy cloned into a λ phage vector, the full-length YFV cDNA was still genetically unstable. Full-length YFV cDNA templates from which infectious RNA could be transcribed were obtained by in vitro ligation of subgenomic cDNA clones (33). Lai et al. (18) reported construction of a full-length cDNA clone of dengue type 4 virus in a plasmid vector. However, plaques produced by recombinant virus are smaller than those of the parent virus (17a). This suggests that mutations that are not lethal for virus replication have been introduced into the dengue type 4 virus cDNA during replication as plasmid DNA. We have prepared a full-length clone of the JEV genome by ligating the subgenomic viral cDNA clones M343 and H756 into pBR322. Transcripts from this cloned full-length cDNA, which was replicated as a plasmid, were not infectious. From our results, and those of Rice et al. (29), we conclude that even though infectious flavivirus RNA can be transcribed from cloned full-length cDNA, there are risks of introducing mutations into the cDNA during replication of the plasmid in *E. coli*. When cloned full-length cDNA is used for molecular and genetic analyses of virus biology, it must be sequenced to confirm that mutations have not been introduced during plasmid replication. For this reason, we prefer to use in vitro-ligated cDNA templates that are genetically stable, rather than a cloned full-length template, to avoid the risk of spontaneous mutations during plasmid replication. The ability to synthesize infectious JEV RNA from in vitro-ligated cDNA templates will be very useful for molecular and genetic studies of flavivirus molecular biology and pathogenesis.

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