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Mutagenesis of the Signal Sequence of Yellow Fever Virus prM Protein: Enhancement of Signalase Cleavage In Vitro Is Lethal for Virus Production

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Proteolytic processing at the C-prM junction in the flavivirus polyprotein involves coordinated cleavages at the cytoplasmic and luminal sides of an internal signal sequence. We have introduced at the COOH terminus of the yellow fever virus (YFV) prM signal sequence amino acid substitutions (VPQAQA mutation) which uncoupled efficient signal peptidase cleavage of the prM protein from its dependence on prior cleavage in the cytoplasm of the C protein mediated by the viral NS2B-3 protease. Infectivity assays with full-length YFV RNA transcripts showed that the VPQAQA mutation, which enhanced signal peptidase cleavage in vitro, was lethal for infectious virus production. Revertants or second-site mutants were recovered from cells transfected with VPQAQA RNA. Analysis of these viruses revealed that single amino acid substitutions in different domains of the prM signal sequence could restore viability. These variants had growth properties in vertebrate cells which differed only slightly from those of the parent virus, despite efficient signal peptidase cleavage of prM in cell-free expression assays. However, the neurovirulence in mice of the VPQAQA variants was significantly attenuated. This study demonstrates that substitutions in the prM signal sequence which disrupt coordinated cleavages at the C-prM junction can impinge on the biological properties of the mutant viruses. Factors other than the rate of production of prM are vitally controlled by regulated cleavages at this site.

Yellow fever virus (YFV) is the type species of the *Flaviviridae*, a family of enveloped, positive-strand RNA viruses. The virus is transmitted by the bite of infected mosquitoes to humans and nonhuman primate hosts. Infection of humans with YFV frequently results in a severe illness characterized by hemorrhagic fever and liver pathology. A highly efficient live attenuated vaccine against yellow fever, strain 17D, has been available for approximately 50 years. Nevertheless, yellow fever continues to be a public health problem in tropical and subtropical regions of the Americas and Africa (reviewed in reference 15).

The YFV 17D genome is comprised of 10,862 nucleotides and is translated into a single polyprotein (23). The polyprotein precursor traverses the membrane of the endoplasmic reticulum (ER) multiple times and is proteolytically processed to at least 10 viral proteins (reviewed in reference 21). The structural proteins, capsid (C), precursor to membrane (prM), and envelope (E), are located in the NH_2-terminal one-quarter of the polyprotein, followed by the nonstructural (NS) proteins NS1 to NS5. The cellular enzyme, signal peptidase, and the virus-encoded serine protease, NS2B-3, catalyze cleavages of the polyprotein precursor in the lumen of the ER and in the cytoplasm, respectively. A third protease, putatively located inside the ER, is required for processing at the COOH terminus of the flavivirus protein NS1 (5, 18).

Proteolytic processing at two internal signal sequences in the flavivirus polyprotein, at the junctions of the C-prM and NS4A-NS4B proteins, is regulated such that luminal signal peptidase cleavage occurs efficiently only after cleavage upstream of the signal sequence mediated by the cytoplasmic viral protease (1, 10, 11, 30). Thus, coexpression of the viral NS2B-3 protease in *cis* or in *trans* with the structural polyprotein region of a number of flaviviruses or a YFV polyprotein fragment encompassing NS4A and NS4B greatly enhanced the efficiency of signal peptidase cleavage at the NH_2 termini of prM and NS4B, respectively. This mechanism for control of the catalytic activity of signal peptidase was unexpected in view of the rapid, mostly cotranslocational processing at signal peptidase cleavage sites (3, 20) and has been described only for the processing of flavivirus polyproteins.

Signal peptides have a low degree of sequence conservation but have common structural motifs (reviewed in references 4, 27, and 28). Amino acids with basic side chains are characteristically located in the NH_2-terminal region of the signal peptide and are a major determinant of the transmembrane topology of integral membrane proteins. The central core region is variable in length (between approximately 7 and 16 residues) and rich in apolar amino acids. The COOH-terminal cleavage region (c-region) must conform to the requirement of small residues, such as alanine, in positions −1 and −3 upstream of the cleavage site and must be in an extended conformation. The c-region, typically 6 amino acids in length, frequently contains amino acids with polar side chains and residues with alpha-helix-breaking properties (proline, glycine, or serine) at its boundary with the hydrophobic core (9, 26). We noted that the c-regions of the signal sequences of flavivirus prM proteins are atypically nonpolar and demonstrated that mutations in the c-region of the Murray Valley encephalitis (MVE) virus prM signal sequence which introduced residues typically found in this region greatly increased the extent of cleavage of prM during recombinant expression in the absence of the viral protease (24). Interestingly, the lack of residues with polar side

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chains in the c-region of the signal sequence of the NS4B protein is also common to all flaviviruses and may have a role in maintaining the dependence of signal peptidase cleavage at the NS4A-NS4B junction on prior cleavage at the COOH terminus of NS4A.

To investigate the biological role of the sequential order of cleavages at the flavivirus C-prM junction, we introduced at the COOH terminus of the YFV prM signal sequence a mutation which uncoupled efficient signal peptidase cleavage of the prM protein from the prerequisite of prior cleavage of the C protein by the viral protease in vitro. The effect on virus replication of this prM signal sequence mutation, when incorporated into the full-length YFV genome, was studied.

MATERIALS AND METHODS

Virus and cells. The 17D strain of YFV was from the American Type Culture Collection and was plaque purified twice (13). Working stocks were culture supernatants from infected BHK cells harvested at about 72 h postinfection (p.i.); titers were $\sim 2 \times 10^7$ PFU/ml, as determined by plaque formation on Vero cells. BHK, Vero, CV1, and COS-7 cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal calf serum (FCS). C6/36 cells were maintained in EMEM plus 8% FCS.

Plaque purification. Vero cell monolayers under an agar overlay (1% agar in EMEM plus 2% FCS) were stained with neutral red (0.03% in 0.7% agar) for 12 to 16 h for plaque visualization. Plaques were picked by piercing through the agar with a pipette and rinsing the area of the monolayer with Hanks' balanced salt solution (pH 8.0). Virus recovered was amplified first on C6/36 cells and again on BHK cells. Titers were between 2×10^5 and 3×10^6 PFU/ml.

Plasmid constructs. Phagemid pBSIISK(−)/YFS(+) contains cDNA for the YFV 17D structural polyprotein region (the C-prM-E coding sequence with a TAA termination codon adjacent to the 3′ end of the E protein gene; A. Grakoui and C. M. Rice, unpublished data). The YFV cDNA was subcloned as a *Bam*HI/*Not*I fragment from this phagemid into the eukaryotic expression plasmid pcDNA1 (Invitrogen) to create pYFs. For mutagenesis, a 1,227-bp *Bam*HI/*Sph*I cDNA fragment encompassing the C and prM protein genes was cloned into M13mp18. In vitro site-directed mutagenesis to alter the C-terminal 6 residues of the prM signal sequence from Leu-Leu-Met-Thr-Gly-Gly to Val-Pro-Gln-Ala-Gln-Ala was performed as described previously (24) with oligonucleotide 226 (5′ CAC CAA GGT CAC TGC TTG CGC CTG CGG TAC CAT TCC CAA AAT TAG 3′). The prM signal sequence mutation was introduced into plasmid pYFs by exchanging a 505-bp *Bam*HI/*Sca*I fragment with the fragment encompassing the mutation to generate plasmid pYF.VPQAQA.

Derivatives of pYF5′3′IV (22) containing mutations in the prM signal sequence were made by exchanging a 322-bp *Msc*I/*Nde*I fragment or a 214-bp *Msc*I/*Sca*I fragment with a fragment encompassing the mutations.

VV recombinants. cDNA for the YFV C, prM, and E protein genes, with or without codons for the prM signal sequence mutation, was subcloned as a 2,355-bp *Xba*I fragment from plasmid pYF.VPQAQA or pYF.s, respectively, into the vaccinia virus (VV) recombination plasmid p7.5K.131a. Homologous recombination with VV (strain WR) and plasmid DNAs and with VV-ts7 as a helper virus and bromodeoxyuridine selection were done as described previously (12). VV stocks were crude CV1 cell lysates.

Transient expression in COS-7 cells. COS-7 cells were transfected with 1 to 2 μg of plasmid DNA as previously described (12). Metabolic labeling of proteins, immunoprecipitation, electrophoresis, and fluorography were done as described previously (24).

Cell-free translation. Plasmids pYFs and pYF.VPQAQA were subjected to cell-free translation by use of a TNT rabbit reticulocyte lysate-coupled transcription-translation system with T7 RNA polymerase (Promega) according to the manufacturer's instructions. Canine pancreatic microsomal membranes (5 eq in each 25-μl reaction; Promega) and [^{35}S]PRO-MIX cell-labeling mixture (200 μCi/ml; Amersham) were included in the translation mixture. Alternatively, plasmids were linearized with appropriate restriction enzymes, extracted with phenol-chloroform, precipitated, and transcribed with T7 RNA polymerase (Promega) to generate RNA coding for the YFV structural proteins. The transcription mixture contained 1 mM m^7G (5′)ppp(5′)G cap analogue (Pharmacia); 1 mM each ATP, CTP, and UTP; 100 μg of bovine serum albumin per ml; 5 mM dithiothreitol; 20 mM Tris-HCl [pH 7.6]; 6 mM $MgCl_2$; 2 mM spermidine; 1 U of RNase inhibitor (Pharmacia) per μl; and 0.8 U of T7 RNA polymerase per μl. After incubation at 37°C for 5 min, GTP was added to 1 mM, and incubation was continued for 1 h. RNA transcripts, extracted twice with phenol-chloroform and precipitated with sodium acetate and ethanol, were translated by use of a nuclease-treated rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions. Microsomal membranes and the cell-labeling mixture were added as described above. Translation products were diluted in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 20 μg of phenylmethylsulfonyl fluoride per ml) and incubated at 4°C overnight with anti-YFV hyperimmune ascitic fluid before immunoprecipitation and electrophoresis.

Nucleotide sequence analysis. Total RNA from YFV-infected cells was extracted with a mixture of guanidinium thiocyanate and phenol (29). cDNA was generated with Expand reverse transcriptase (Boehringer Mannheim Biochemicals) and random hexamers, and the region encoding the YFV C and prM proteins was amplified by PCR with an Expand high-fidelity PCR system (Boehringer), a forward primer (5′ CGG GAA GCT TGA GCG ATT AGC AGA GAA CTG ACC 3′), and a reverse primer (5′ CAC TAT TGA TGC AAG CTT CAC AGG 3′) flanking the C and E protein genes. PCR products were gel purified after *Hin*dIII digestion and cloned into *Hin*dIII-linearized, dephosphorylated pBluescript KS(+) (Stratagene). Plasmid DNA was extracted with a Wizard Plus SV Minipreps Kit (Promega) and sequenced with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to manufacturer instructions. M13 forward, M13 reverse, or YFV-specific (5′ GGC TTG GCT GTT CTA AGG 3′) primers were used for sequencing. When viral sequences from electroporated BHK cells were determined without plaque isolation, RNA was extracted from cells infected with BHK cell supernatants collected from transfection experiments and cDNA was generated by reverse transcriptase PCR as described above. Appropriate DNA fragments were gel purified without exposure to UV light and sequenced as described above.

In vitro synthesis and transfection of YFV full-length RNA. The procedures for generating YFV full-length RNA were as described previously (22), with minor modifications. Plasmids pYFM5.2 and pYF5′3′IV (or its mutant derivatives) comprise the central coding region (6.3 kb) and the 5′ (2.3 kb) and 3′ (2.5 kb) coding regions of the YFV genome, respectively (22). These were digested with restriction enzymes *Aat*II and *Apa*I, and fragments of 6.8 and 6.1 kb, respectively, were isolated following agarose gel electrophoresis without exposure of the DNA bands to UV light. Equimolar ratios of the two fragments were ligated to generate full-length cDNA templates, which were transcribed with SP6 RNA polymerase (Promega) as described above. For quantitation of full-length RNA, the transcription mixtures were electrophoresed in 1% agarose gels containing 0.1% sodium dodecyl sulfate (SDS).

RNA transcripts were introduced into BHK cells by electroporation. BHK cells from subconfluent monolayers were washed twice and suspended at 1.25×10^7 cells/ml in serum-free EMEM (GibcoBRL). Cells (10^7) were mixed with RNA transcripts at room temperature in an electroporation chamber (standard 0.4-cm gap; GibcoBRL) and subjected to two consecutive pulses at 250 V, 800 μF, and the low-ohm setting of Cell-Porator Electroporation System I (GibcoBRL). Cells were left at room temperature to recover for 5 min, mixed with 24 ml of growth medium, transferred to culture dishes, and incubated at 37°C.

Infectious-center assay. Ten-fold dilution series of electroporated cells were prepared by use of growth medium containing 4×10^5 freshly harvested BHK cells per ml. Diluted samples (1 ml/well) were transferred to six-well dishes and incubated at 37°C. Culture medium was removed, and an agar overlay (1% agar in EMEM plus 2% FCS) was added to cell monolayers between 6 and 18 h after transfection. For visualization of plaques, the agar overlay was removed 3 to 5 days after transfection, and cell monolayers were stained with crystal violet (0.1% in 20% ethanol).

Immunofluorescence staining. Electroporated BHK cells were plated in 24-well trays (4×10^4 cells/well). At 6 h posttransfection, the culture medium was removed, fresh growth medium with or without ammonium chloride (50 mM) was added, and incubation was continued at 37°C. At 42 h posttransfection, monolayers were washed once with phosphate-buffered saline (PBS) and fixed for 1 min in a mixture of ice-cold acetone and methanol (1:1). Cells were incubated with anti-YFV hyperimmune ascitic fluid (diluted in PBS plus 2% FCS) at 37°C for 1 h, washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (Selenius; diluted 1:300 in PBS plus 2% FCS) at 37°C for 30 min.

Virulence assay. The neurovirulence of YFV 17D and variants was assayed in pathogen-free 4-week-old outbred Swiss mice (obtained from the Animal Breeding Facility at the John Curtin School of Medical Research, The Australian National University, Canberra, Australian Capital Territory, Australia) essentially as described previously (16). Groups of animals were inoculated by the intracerebral (i.c.) route with 10^3 or 10^4 PFU of virus from stocks titrated by plaque formation on Vero cells. Mouse survival data were analyzed by the two-sided Fisher exact test.

RESULTS

Mutations in the c-region of the prM signal sequence augment the production of prM during recombinant expression of the YFV structural polyprotein. The flavivirus C-prM junction spans from the cytosolic cleavage site at the C terminus of the C protein to the ER luminal signal peptidase cleavage site at the N terminus of prM and encompasses 20 amino acids which transverse the ER membrane (see Table 2). Expression of the flavivirus structural polyprotein region (encompassing the C, prM, and E proteins) in the absence of coexpression of the

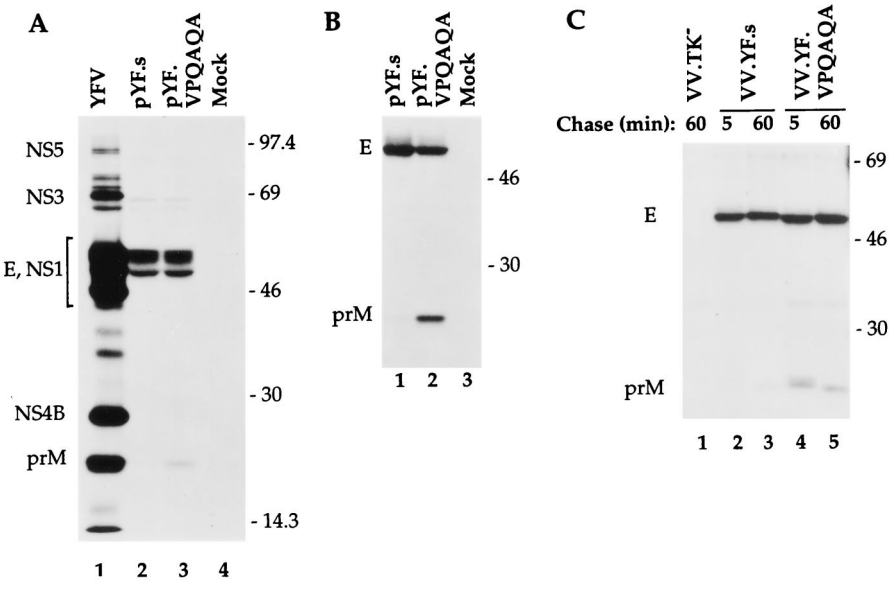

FIG. 1. Effect of the VPQAQA mutation in the signal sequence of prM on the efficiency of cleavage of prM in cell-free and transient expression assays. (A) COS-7 cells were transfected with eukaryotic expression plasmids encoding the YFV C, prM, and E proteins and with (pYF.VPQAQA) or without (pYF.s) the prM signal sequence mutation, infected with YFV (MOI, ~10), or left untreated. At 2 days after transfection and 20 h after infection, the cells were metabolically labeled for 30 min and then chased for 1.5 h. Immunoprecipitation was done with anti-YFV hyperimmune ascitic fluid, and proteins were separated by SDS-PAGE (10% acrylamide). (B) Cell-free translation of YFV structural proteins was performed in the presence of microsomal membranes. Immunoprecipitation was done as described above, and proteins were analyzed by SDS-PAGE (12% acrylamide). Lanes 1 and 2 show translation products obtained with RNA transcribed in vitro from pYF.s and pYF.VPQAQA, respectively, and lane 3 shows a control transcription-translation-translocation reaction performed in the absence of plasmid DNA. (C) For VV vector expression of the YFV structural proteins with (VV.YF.VPQAQA) or without (VV.YF.s) the prM signal sequence mutation, CV1 cells were infected for 3 h (MOI, ~10), starved for 30 min in methionine-free medium, and pulse-labeled for 15 min. The label was chased for the indicated times, and YFV proteins were immunoprecipitated and analyzed by SDS-PAGE (12% acrylamide). VV.TK$^-$ is a control virus which has no foreign DNA insert. Bands corresponding to YFV proteins are labeled on the left and sizes (in kilodaltons) of marker proteins are shown on right of the autoradiograms.

viral protease, NS2B-3, results in inefficient signal peptidase cleavage at the C-prM junction (1, 11, 30). This fact is evidenced by a much reduced production of prM in comparison to that of the E protein. To test whether signal peptidase cleavage at the YFV C-prM junction can be removed from its dependence on prior cytoplasmic cleavage of the C protein by the viral protease, the c-region of the prM signal sequence was changed from Leu-Leu-Met-Thr-Gly-Gly to Val-Pro-Gln-Ala-Gln-Ala (VPQAQA mutation). The latter sequence is analogous to an idealized signal sequence c-region which, when substituted into the MVE virus structural polyprotein, significantly increased the extent of cleavage of prM (24). The mutation increased the cleavage potential score at the NH_2 terminus of YFV prM (based on the weight-matrix algorithm of von Heijne and determined by a computer program [8]) from 2.94 to 7.60. Importantly, the amino acid substitutions did not introduce putative alternative signal peptidase cleavage sites, according to the "−1,−3 rule" for signal peptidase recognition.

The results of experiments done with three different expression systems for the synthesis of the YFV structural polyprotein with or without the prM signal sequence mutation are shown in Fig. 1. In COS-7 cells, prM was immunoprecipitated from lysates of cells transfected with pYF.VPQAQA but was not detected in lysates of cells transfected with the parent plasmid pYF.s, while comparable amounts of the E protein were seen (Fig. 1A, lanes 2 and 3). The anti-YFV hyperimmune serum recognized other viral proteins in addition to the E protein and prM. NS5, NS3, NS1, and NS4B are identified on the profile for YFV-infected COS-7 cells (Fig. 1A, lane 1).

The results of an in vitro transcription-translation-translocation experiment with the two plasmids encoding the parent and mutated YFV structural polyprotein regions are shown in Fig. 1B. prM could barely be detected among translation products from pYF.s (Fig. 1B, lane 1), whereas a much larger amount was produced from pYF.VPQAQA (lane 2), even though both reaction mixtures contained similar levels of the E protein. No difference in electrophoretic mobility was seen between the prM proteins encoded from the two constructs, implying that cleavage had occurred at the authentic NH_2 terminus of prM synthesized from pYF.VPQAQA. The E and prM protein-specific bands were not seen in the profile of a control reaction mixture containing no DNA (Fig. 1B, lane 3).

VV recombinants encoding the YFV C, prM, and E proteins with (VV.YF.VPQAQA) or without (VV.YF.s) the prM signal sequence mutation were used to infect CV1 cells in a pulse-chase experiment to compare the efficiencies of production of prM. Following a 15-min labeling interval and a 5-min chase period, prM was not detectable in immunoprecipitates of VV.YF.s-infected cells but was clearly visible in the protein profile for VV.YF.VPQAQA-infected cells (Fig. 1C, lanes 2 and 4). After a 1-h chase, prM proteins with similar electrophoretic mobilities were precipitated from lysates of cells infected with either of the two VV recombinants. The efficiency of production of prM was, however, greater in the presence of the signal sequence mutation (Fig. 1C, lanes 3 and 5). Similar amounts of the E protein were immunoprecipitated from VV.YF.s- and VV.YF.VPQAQA-infected cell lysates, and no bands were detected in lysates from cells infected with the control virus, VV.TK$^-$ (Fig. 1C, lane 1). prM in VV.YF.VPQAQA-infected cells migrates as a doublet during SDS-polyacrylamide gel electrophoresis (PAGE), and the slower-migrating form is chased to the faster-migrating form. This finding is consistent with the maturation of carbohydrates on YFV prM (2). In summary, our findings confirm that hydro-

TABLE 1. Effect of mutations in the c-region of the signal sequence of YFV prM on virus replication

Expt	RNA (μg)[c]	Infectious centers from[a]:		Titer (PFU/ml) of[b]:	
		17D	VPQAQA variant	17D	VPQAQA variant
1	1	NT[d]	<25	5×10^6	60
2a	0.9	5×10^4	<25	6×10^6	3×10^2
2b	0.09	2.5×10^3	<25	NT	<30
3	1	2.5×10^4	<25	3×10^6	2×10^2
4	5	5×10^4	<25	9×10^6	<10
5	1	7.5×10^4	<25	5×10^6	2×10^3

[a] Number of transfected cells yielding plaques in an infectious-center assay (see Materials and Methods), calculated for 10^7 cells electroporated with the indicated amount of RNA.

[b] Virus titers in culture fluids from dishes containing 2×10^6 electroporated cells at 65 to 79 h after electroporation were determined by plaque formation on Vero cells.

[c] Amount of RNA used in electroporation of 10^7 BHK cells.

[d] NT, not tested.

phobic residues in the c-region of the YFV prM signal sequence play a role in markedly reducing the accessibility of the signal peptidase cleavage site for recognition by the signal peptidase.

Mutations in the signal sequence of prM which enhance signal peptidase cleavage in vitro are detrimental for YFV replication. The conservation among flaviviruses of the dependence on prior cleavage of the C protein for efficient processing of the signal sequence of prM implies a functional role in flavivirus replication (24). To investigate the biological significance of the coordinated cleavages at the C-prM junction, codons for the VPQAQA mutation were incorporated upstream of the prM signal peptidase cleavage site into the YFV 17D genome transcribed in vitro from full-length cDNA. The mutation alters the prM signal peptide such that it is putatively more favorable for recognition by the signal peptidase at the authentic NH_2 terminus of prM. Table 1 shows the results of six transfections of BHK cells with in vitro-synthesized parent (17D) or prM signal sequence mutant (VPQAQA) RNA. In each of the experiments, the presence of the prM signal sequence mutation abolished or severely reduced the production of plaque-forming YFV. In contrast, transfection with 17D RNA reliably produced progeny virus. Thus, when serial dilutions of transfected cells were plated with an agar overlay, plaques were always visible at 3 days after electroporation with in vitro-synthesized 17D RNA but were never apparent after electroporation with VPQAQA RNA. Small differences in the amounts of parent and mutant RNAs used in transfections could not have accounted for this difference in infectious-center formation, since a 10-fold reduction of 17D RNA in parallel electroporations did not abolish but reduced the formation of infectious centers by a similar magnitude (Table 1, experiment 2).

Despite the failure of the production of infectious centers in BHK cells transfected with VPQAQA RNA, virus could be detected in culture fluid harvested at approximately 72 h after electroporation and used in plaque titrations on Vero cell monolayers (Table 1). However, the virus titers in culture fluids from cells transfected with VPQAQA RNA were always significantly lower (10^3- to 10^5-fold) than those in culture fluids from cells transfected with 17D RNA. The production of infectious virus from transfection with VPQAQA RNA was confirmed by immunofluorescence staining and radioimmunoprecipitation with YFV-specific antibodies in BHK cells infected with culture fluids harvested from transfected cells 3 days after electroporation (data not shown). From these data, it is apparent that the mutation in the prM signal sequence severely restricted or prevented virus production. In the latter case, it would be necessary to postulate that the production of low titers of infectious virus was the consequence of reversion or second-site mutation events (see below).

The prM signal sequence mutation does not prevent virus-specific protein synthesis following transfection with in vitro-synthesized RNA. To investigate whether the mutation in the prM signal sequence influenced the early events of viral macromolecular synthesis or the later stages of assembly and maturation during virus replication, the kinetics of virus-specific protein synthesis in cells transfected with in vitro-synthesized 17D or VPQAQA RNA were examined. Virus-infected, transfected, or untreated cell monolayers were metabolically labeled, and cell lysates were subjected to immunoprecipitation with an anti-YFV hyperimmune serum. Virus-specific proteins could already be detected when cells were pulse-labeled from 12 to 15 h after electroporation with either 17D or VPQAQA RNA (Fig. 2A, lanes 3 and 4). Bands corresponding to the NS3 (~69 kDa), E (~50 kDa), and NS1 (~48 kDa) proteins were apparent, and these were not seen in mock-treated controls (Fig. 2A, lanes 2, 5, and 8). For comparison, a protein profile of the immunoprecipitate from cells infected with YFV and labeled from 12 to 15 h after infection is shown (Fig. 2A, lane 1). Virus infection resulted in a much greater amount of viral protein synthesis at this early time point than was seen with RNA transfections, presumably due to the much larger number of cells that were infected (multiplicity of infection [MOI], ~1) than of cells that could be transfected. When transfected cells were pulse-labeled from 18 to 21 h after electroporation, a large increase in the amount of virus-specific protein synthesis was noted compared to that seen during the earlier labeling period. In addition to the three large proteins (NS3, E, and NS1), the NS4B (~28 kDa) and NS2B (~14 kDa) proteins were also apparent. The prM protein was visible as a strongly labeled doublet (~24 kDa) in the protein profiles corresponding to cells transfected with 17D RNA but not in those corresponding to cells transfected with VPQAQA RNA (Fig. 2A, compare lanes 6 and 7). This result was inconsistent with the relative differences in the amounts of other radiolabeled YFV-specific proteins found in cells transfected with 17D or VPQAQA RNA and appears to contradict the enhancement of prM production seen in vitro as a result of the prM signal sequence mutation. The discrepancy most likely reflects the instability or inefficient recovery of prM from Nonidet P-40 lysates with the anti-YFV immune serum. The antigenicity or stability of prM may be increased following heterodimerization with the E protein and/or assembly into particles. This assembly process may not take place efficiently in the presence of the prM signal sequence mutation. The synthesis of YFV-specific proteins in cells transfected with 17D RNA further increased between 36 and 39 h after electroporation in comparison to that seen at 18 to 21 h after transfection. This increase was not apparent for most proteins in cells transfected with VPQAQA RNA (Fig. 2A, lanes 9 and 10). It is indicative of a second round of infection in monolayers transfected with 17D RNA which does not occur to a significant extent following transfection with VPQAQA RNA (see below).

Our results clearly show that viral protein synthesis and, accordingly, viral RNA synthesis are not severely inhibited by the VPQAQA mutation, although at all three time points examined viral protein synthesis in cells transfected with 17D RNA was greater than that in cells transfected with the mutant RNA. Growth curves showing virus release into culture fluids

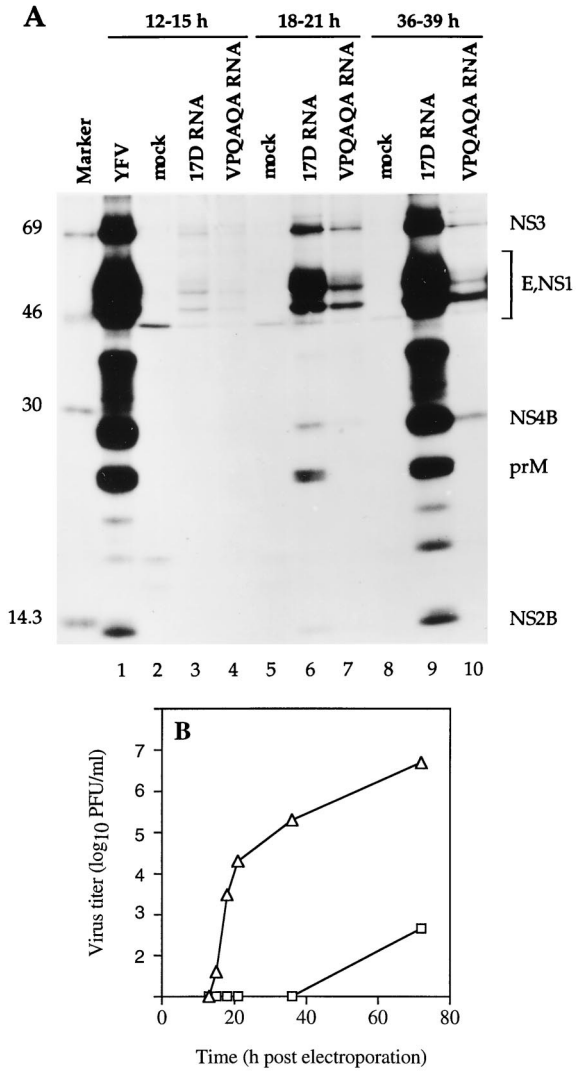

FIG. 2. YFV protein synthesis and viral replication in BHK cells transfected with in vitro-synthesized YFV 17D or VPQAQA RNA. BHK cells (10^7) were electroporated with approximately 1 μg of 17D or VPQAQA RNA and seeded in 60-mm petri dishes (2×10^6 cells/dish) for the kinetic study of virus-specific protein synthesis and virus replication. (A) Electroporated, YFV-infected (MOI, ~1), or untreated cells were metabolically labeled for 3 h at various times after infection or transfection as shown. Immunoprecipitation was performed with YFV-specific hyperimmune ascitic fluid, and proteins were analyzed by SDS-PAGE (12% acrylamide). Sizes (in kilodaltons) of marker proteins are shown on the left, and viral proteins are labeled on the right. (B) Aliquots were taken at the indicated times from the culture supernatants of cells electroporated with 17D (triangles) or VPQAQA (squares) RNA, and virus titers were determined by plaque formation on Vero cells.

of cell aliquots from the same electroporation as that used in the kinetic study of viral protein synthesis are shown in Fig. 2B. Virus release from cells transfected with 17D RNA first occurred at 15 h and increased exponentially until 72 h after electroporation. In contrast, transfection with the mutant RNA did not result in detectable virus release in the first 36 h and only low virus titers at 72 h after electroporation, consistent with the results shown in Table 1. Accordingly, it appears that a stage in virus assembly or maturation leading to the release of infectious virus progeny is inhibited by the VPQAQA mutation in the signal sequence of prM. To rule out the possibility that noninfectious particles were released from cells transfected

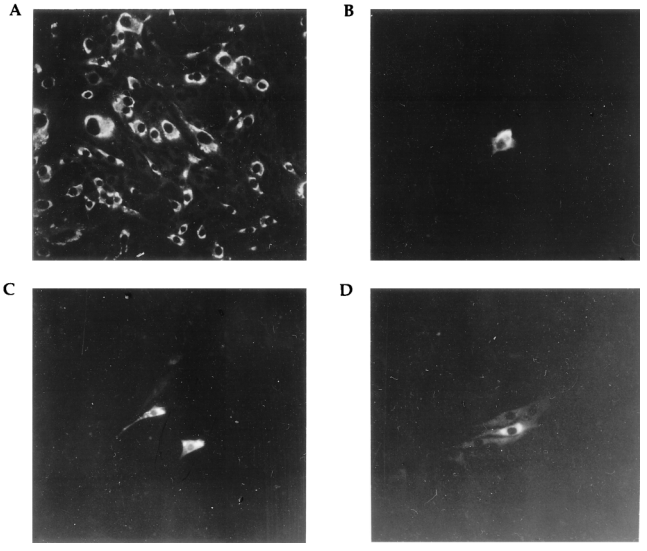

FIG. 3. Detection of YFV proteins in BHK cells transfected with 17D or VPQAQA RNA. BHK cells were electroporated with approximately 0.1 μg of 17D (A and B) or VPQAQA (C and D) RNA and seeded in a 24-well tray (4×10^4 cells/well). After incubation at 37°C for 6 h, culture medium from each well was replaced with fresh medium with (B and D) or without (A and C) ammonium chloride. Monolayers were fixed at 42 h posttransfection, and an immunofluorescence assay was performed with anti-YFV hyperimmune ascitic fluid. Areas in each well with the greatest density of fluorescent cells are shown.

with VPQAQA RNA, we performed reverse transcriptase PCR on material recovered by polyethylene glycol concentration from culture supernatants. At 24 h after electroporation, YFV-specific sequences could be amplified from culture fluids of cells transfected with 17D RNA but not from those of cells transfected with VPQAQA RNA (data not shown).

Suppression of viral progeny but not antigen production by the prM signal sequence mutation. BHK cells were transfected with equal amounts of in vitro-synthesized 17D or VPQAQA RNA by electroporation and grown in the presence or absence of NH_4Cl to inhibit the release of infectious virus from the transfected cells and, in turn, the initiation of second-round infections. NH_4Cl is an acidotropic agent which prevents the cleavage of prM in the trans-Golgi network by a cellular protease and hence activation of the infectivity of released virions (6, 7, 19). The concentration of NH_4Cl used prevented the release of infectious virus from BHK cells infected with YFV (MOI, ~1) over a period of 48 h without suppressing the accumulation of viral antigens in the infected cells or inducing detectable cytopathology (data not shown). At 42 h after electroporation, immunofluorescence staining for YFV-specific antigens was performed. On monolayers transfected with 17D RNA and grown in the absence of NH_4Cl, multiple fluorescent foci corresponding to YFV-infected cells were apparent (Fig. 3A). In contrast, transfection with the prM signal sequence mutant RNA yielded only individual fluorescent cells (Fig. 3C), demonstrating that YFV-specific antigens were produced in the transfected cells but apparently in the absence of the production of viral progeny and the establishment of second-round infections. This result was confirmed when cells transfected with 17D or VPQAQA RNA were grown in the presence of NH_4Cl (Fig. 3B or D, respectively). For both RNAs, similar numbers of single fluorescent cells were seen (~$20/10^5$ electroporated BHK cells), consistent with comparable transfection efficiencies for the RNAs.

TABLE 2. Amino acid changes in the prM signal sequence of virus recovered from transfections with VPQAQA RNA

Virus	Expt[a]	Mutation[b]	Deduced amino acid sequence at the C-prM junction[c]
			↓ ↓
VPQAQA			SRKRR SHDVLTVQFLILGMVPQAQA VTLVR
V1(H_{103}→Y, D_{104}→G)	1	H_{103}→Y (CAT→TAT) D_{104}→G (GAT→GGT)	..... .YG..... .....
V2(P_{117}→L)	2	P_{117}→L (CCG→CTG)	....L..... .....
V3(H_{103}→Y)	4	H_{103}→Y (CAT→TAT)	..... .Y..... .....
V4(H_{103}→L)	5	H_{103}→L (CAT→CTT)	..... .L..... .....
V5(P_{117}→A)	5	P_{117}→A (CCG→GCG)	....A..... .....
V6(H_{103}→L)	5	H_{103}→L (CAT→CTT)	..... .L..... .....
V7(T_{107}→I)	5	T_{107}→I (ACT→ATT)	....I..... .....
V8(H_{103}→L)	3	H_{103}→L (CAT→CTT)	..... .L..... .....
V9(H_{103}→L)	1	H_{103}→L (CAT→CTT)	..... .L..... .....
V10(Q_{109}→L)[d]	4	Q_{109}→L (CAT→CTT)	....L..... .....
17D			....LLMTGG .....

[a] Experiments are numbered as in Table 1.
[b] Amino acids are numbered from the NH_2-terminal residue in the YFV polyprotein; corresponding codons are shown in parentheses.
[c] The amino acid sequence begins with the five residues NH_2 terminal to the viral protease cleavage site at the C-prM junction and ends with the five residues COOH terminal to the signal peptidase cleavage site. Arrows indicate the two cleavage sites; dots indicate identities with the VPQAQA mutant sequence.
[d] Virus was not plaque purified.

Viral progeny from cells transfected with YFV VPQAQA RNA contain genotypic reversions or second-site mutations. We have shown that transfection of BHK cells with in vitro-synthesized YFV RNA encompassing the VPQAQA mutation in the prM signal sequence results in the release of some viruses into culture fluids, first detectable at about 3 days following electroporation (Table 1 and Fig. 2B). To test whether these viruses had retained the VPQAQA mutation or whether their production required reversions or second-site mutations, the nucleotide sequences of their C and prM protein genes were determined. Viruses in culture fluids harvested on day 3 after transfection with VPQAQA RNA were plaque purified on Vero cells and amplified on mosquito (C6/36) cells for 5 days. Total RNA from infected C6/36 cells was extracted, and the structural polyprotein genes were amplified by reverse transcriptase PCR. Cloned PCR products were sequenced from nucleotides 163 to 990, a region containing the entire C and prM protein genes. Table 2 shows the deduced amino acid sequences at the C-prM junction of 10 virus stocks isolated from five different transfections. In all cases, point mutations which were exclusively located in the prM signal sequence were found, and all gave rise to amino acid changes. These results strongly suggest that the YFV 17D genome containing the VPQAQA signal sequence mutation is replication deficient and that viability can be restored by reversions or second-site mutations of single amino acids in the prM signal sequence. Amino acid substitutions occurred at His_{103}, Asp_{104}, Thr_{107}, Gln_{109}, and Pro_{117} and always involved mutations to residues of greater hydrophobicity. Only 2 of the 10 variants had a change in the mutated c-region of the prM signal sequence involving a substitution of Leu or Ala for Pro_{117}. Surprisingly, substitutions at His_{103}, two residues downstream from the NS2B-3 cleavage site in the C terminus of the C protein, also allowed the production of viable viral progeny despite the presence of the VPQAQA mutation in the c-region of the prM signal sequence. This residue was apparently the preferred target for second-site mutations (6 of 10 variants analyzed) to restore viability to the VPQAQA virus. A third region more centrally located in the prM signal sequence also restored viability to the VPQAQA virus following a point mutation at Thr_{107} to Ile or Gln_{109} to Leu (Table 2). These results raise the question of whether the second-site mutations in the NH_2-terminal and core regions of the prM signal sequence exert an inhibitory influence on the downstream signal peptidase cleavage of prM or if they restore viability by an alternative effect.

Signal peptidase cleavage in vitro at the C-prM junction in VPQAQA variants. To investigate whether the reversions and second-site mutations in the prM signal sequence found in the VPQAQA variants impinge on the efficiency of signal peptidase cleavage of prM, cell-free translation-translocation experiments were performed. RNA transcripts encoding the C, prM, and E proteins of variants V2(P_{117}→L), V4(H_{103}→L), and V7(T_{107}→I), representing the three regions in the prM signal sequence where substitutions were found, were used in these experiments. The cDNA constructs used for RNA transcription were those subjected to sequence analysis. Membrane-associated translation products, adjusted for similar E protein band intensities, are shown in Fig. 4. The protein profiles of each of the three VPQAQA variants demonstrate the efficient production of prM, which was comparable to that seen in the profile for plasmid pYF.VPQAQA and was significantly greater than that seen in the profile for the wild-type plasmid, pYF.s. A protein band with the estimated molecular mass of the C-prM precursor (~34 kDa) was apparent in the translation products from pYF.s upon overexposure of the autoradiogram (data not shown) but was not seen in the presence of the signal sequence mutation or the variants derived from it. Accordingly, the reversions and second-site substitutions do not cause reversion of the signal peptidase cleavage site of prM to a less accessible conformation.

Growth properties of VPQAQA variants. The growth properties in Vero and BHK cells of three revertants or second-site mutants derived from transfections with VPQAQA RNA were compared to those of YFV 17D. Virus stocks were plaque isolates V2(P_{117}→L), V4(H_{103}→L), V7(T_{107}→I), and 17D which had been amplified once in C6/36 cells and once in BHK cells. Extracellular titers from infected Vero and BHK cells (MOI, ~0.1) were assayed between 24 and 72 h p.i. In Vero cells, significantly lower titers (6- to 15-fold) were observed between 30 and 72 h p.i. for variants V4(H_{103}→L) and V7(T_{107}→I) than for YFV 17D. Variant V2(P_{117}→L) showed not more than a threefold difference in growth titer compared with 17D (Fig. 5A). In BHK cells, V2(P_{117}→L), V4(H_{103}→L), and V7(T_{107}→I) also grew to lower titers than 17D; however, the differences were not more than fivefold (data not shown).

To assess the contribution of regions other than the C-prM

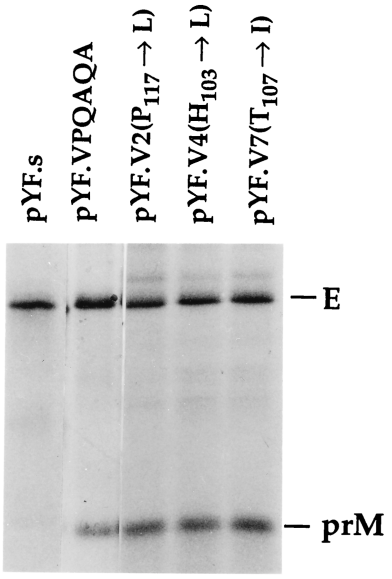

FIG. 4. Effect of reversions and second-site mutations in the prM signal sequence on the efficiency of cleavage of prM in cell-free translation assays. Cell-free translation of YFV structural proteins was performed in the presence of microsomal membranes with appropriate RNA transcripts derived from plasmids pYF.s, pYF.VPQAQA, pYF.V2(P_{117}→L), pYF.V4(H_{103}→L), and pYF.V7(T_{107}→I). Immunoprecipitation was performed with anti-YFV hyperimmune ascitic fluid, and proteins were separated by SDS-PAGE (12% acrylamide). Bands corresponding to YFV proteins E and prM are labeled on right.

junction to the growth properties of the VPQAQA variants, cDNA fragments encoding the C-prM junction (322 bp corresponding to amino acids 89 to 176) of variants V2(P_{117}→L), V4(H_{103}→L), and V7(T_{107}→I) were cloned into the parental YFV plasmid (pYF5′3′IV), and full-length RNA transcripts were produced [designated V2′(P_{117}→L), V4′(H_{103}→L), and V7′(T_{107}→I) RNAs]. BHK cells were electroporated with similar amounts (~0.1 μg) of each transcript, and virus in the culture medium was titrated from 16 to 72 h posttransfection. Virus titers resulting from transfection with V2′(P_{117}→L) or V4′(H_{103}→L) RNA were 4- to 5-fold and 10- to 15-fold lower, respectively, than those resulting from transfection with 17D RNA between 21 and 64 h posttransfection (Fig. 5B). V7′(T_{107}→I) RNA gave rise to titers more than 100-fold lower than those from 17D RNA in the same experiment (Fig. 5B). A comparison of infectious centers resulting from transfection of BHK cells showed no significant difference between 17D, V2′(P_{117}→L), and V4′(H_{103}→L) RNAs; approximately 500 to 1000 infectious centers were obtained per 10^7 transfected cells (data not shown). Infectious centers were not detected following transfection with V7′(T_{107}→I) RNA. These results confirm that the changes at amino acids His_{103} and Pro_{117} were responsible for overcoming the lethal effects of the VPQAQA mutation and exclude the possibility that mutations at other sites in the viral genome were required. However, the lower virus titers produced by variants V2(P_{117}→L) and V4(H_{103}→L) in infected Vero cells and V2′(P_{117}→L) and V4′(H_{103}→L) RNAs in transfected BHK cells, compared to those produced by YFV 17D, suggest that minor defects in replication still exist. Transfection of BHK cells with V7′(T_{107}→I) RNA resulted in a delayed and much lower yield of virus progeny than infection with V7(T_{107}→I) virus stocks. This result suggests that additional mutations, other than the Thr_{107}-to-Ile change, outside the region of amino acids 89 to 176, contribute to the restoration of viability of the latter.

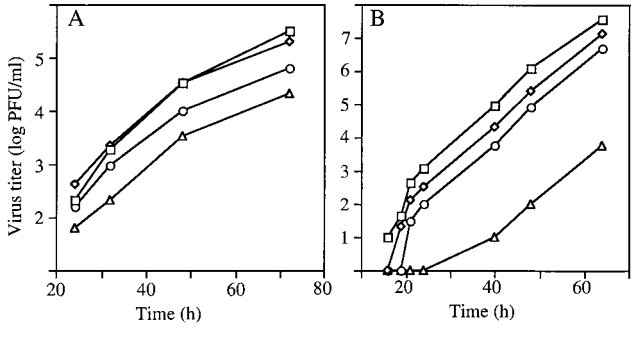

FIG. 5. Virus titers of VPQAQA variants in vertebrate cells. (A) Vero cells (10^5) in 24-well dishes were infected (MOI, ~0.1) with YFV 17D (squares) or VPQAQA variants V2(P_{117}→L) (diamonds), V4(H_{103}→L) (circles), and V7(T_{107}→I) (triangles), and samples were taken between 24 and 72 h p.i. for assay of infectivity titers. (B) BHK cells were electroporated with full-length RNA transcripts (~0.1 μg) incorporating the coding regions for the C-prM junctions of V2(P_{117}→L), V4(H_{103}→L), V7(T_{107}→I), and YFV 17D. Electroporated cells (2×10^6) were plated in 60-mm dishes, and supernatant samples were taken between 16 and 64 h posttransfection for assay of infectivity titers. Data for 17D RNA (squares), V2′(P_{117}→L) RNA (diamonds), V4′(H_{103}→L) RNA (circles), and V7′(T_{107}→I) RNA (triangles) are shown.

TABLE 3. Neurovirulence of YFV 17D and VPQAQA variants in mice

Virus	Dose (PFU)	No. of dead mice/ no tested (%)	Avg time to death (days)
17D	10^4	7/7 (100)	6.9
	10^3	7/7 (100)	7.0
V2(P_{117}→L)	10^4	6/8 (75)	10.5
	10^3	3/7 (43)	13.3
V4(H_{103}→L)	10^4	0/7 (0)	
	10^3	0/5 (0)	

Mouse neurovirulence of VPQAQA variants. The neurovirulence in mice of two variants derived from transfection with VPQAQA RNA, V2(P_{117}→L) and V4(H_{103}→L), was compared to that of YFV 17D (culture fluid from BHK cells transfected with infectious clone cDNA). Groups of mice were inoculated i.c. with 10^3 or 10^4 PFU of the three viruses, and the animals were observed for signs of encephalitis or death (Table 3). YFV 17D caused 100% mortality with both doses; the average time to death was 7 days p.i. Clinical signs of encephalitis were apparent from day 5 p.i. in all animals in the two groups. The reversion of Pro_{117} to Leu in the VPQAQA signal sequence mutation of variant V2(P_{117}→L) resulted in a significant attenuation of neurovirulence in comparison to that of YFV 17D (*P*, 0.07). This result was reflected in a dose-dependent reduction in mortality and an increase in the average time to death. The neurovirulence of variant V4(H_{103}→L) was even further attenuated, with no death or clinical signs of encephalitis resulting from i.c. inoculations with this virus at 10^3 and 10^4 PFU (*P*, 0.0006). Interestingly, this VPQAQA variant also exhibited the slowest growth kinetics in vertebrate cells among the three viruses tested for neurovirulence.

DISCUSSION

We have investigated the role in virus replication of the coordinated sequence of cleavages at the flavivirus C-prM junction. To override the dependence of signal peptidase

cleavage of prM on the prior cytoplasmic cleavage of the C protein by the viral NS2B-3 protease, the c-region of the prM signal peptide was substituted with residues which conform to an idealized signal sequence. This mutation was chosen on the basis of its significant enhancement of prM production in MVE polyprotein expression studies (24). Here we have confirmed a similar effect of increased signal peptidase cleavage of prM during expression of the YFV structural polyprotein region in the presence of the VPQAQA mutation and, in addition, have tested the effect of this mutation on virus production. Our results support the notion that coordinated proteolytic processing at the C-prM junction is at least in part maintained by a feature conserved between flaviviruses at the C terminus of the signal peptide. This region characteristically lacks polar residues and is predicted to impose an alpha-helical rather than the extended beta-strand conformation required for interaction of the signal peptidase cleavage domain with the active site of the enzyme (17, 25). As a consequence, the flavivirus prM signal peptide is predicted to extend across the lipid head group region into the lumen of the ER, similar to transmembrane helices of integral membrane proteins, and to maintain the signal peptidase cleavage site in a cryptic conformation. A further requirement for such a model for the coordination of cleavage between the C protein and prM is the prediction that the cleavage of the C protein by the viral protease will elicit slippage of the signal peptide back and forth in the translocation channel due to Brownian motion (18a) which, in turn, will trigger the cleavage of prM by the signal peptidase.

The VPQAQA mutation replaces the 6 COOH-terminal amino acids of the YFV signal sequence with residues that are frequently found at the corresponding positions in natural eukaryotic signal sequences. This fact is reflected in a significant improvement in the predicted cleavage score, based on a weight-matrix algorithm (8), at the authentic prM signal peptidase cleavage site without the production of alternative sites in the vicinity of the C-prM junction. Our data are consistent with the synthesis of authentic prM from the mutated constructs, based on the electrophoretic mobility of prM. In addition, revertants and second-site mutants selected from transfections with VPQAQA RNA did not reveal amino acid substitutions which resulted in predicted alternative signal peptidase cleavage sites for prM. We can also discount the possibility that the VPQAQA mutation influenced the membrane topology or translocation efficiency of prM and the downstream transmembrane proteins, since three different expression systems yielded, reproducibly, comparable amounts of the E protein from the YFV wild-type and mutated structural polyprotein constructs. The production of the E protein from the polytopic polyprotein fragment requires the correct membrane topology of the transmembrane segments (14). We conclude that the primary effect of the VPQAQA mutation is the enhancement of signal peptidase cleavage of prM without alteration of the cleavage site or membrane topology of the polyprotein.

A remarkable finding of this study is the observation that a mutation in the YFV prM signal peptide which enhances cleavage by signal peptidase almost totally suppresses infectious virion production. The signal sequence for prM functions essentially only in protein translocation and cleavage and is not expected to be a component in the assembly of virus particles. Thus, our results provide strong evidence in favor of a biological role for the down-regulation of signal peptidase cleavage at the C-prM junction prior to processing of the C protein by the NS2B-3 protease. We have excluded the possibility that the prM signal sequence mutation abolishes the early events of viral replication (viral RNA and protein syntheses) but have not resolved which later stage of the viral life cycle is inhibited. A consequence of the prM signal sequence mutation may have been the inhibition of flavivirus assembly, which takes place on intracellular membranes probably derived from the ER. The events following the dimerization of prM and the E protein, which lead to the formation of enveloped viral particles, remain undefined, and assembly intermediates such as nucleocapsid precursors or budding structures on intracellular membranes have not been detected (21). Flavivirus mutants with putative assembly defects would, therefore, be valuable tools for unraveling this process in biochemical and ultrastructural studies.

We have isolated a number of revertants and second-site mutants from BHK cells transfected with VPQAQA RNA. All viruses derived from transfections with the mutated RNA had amino acid changes in the prM signal sequence. Nearly all of these changes involved the substitution of a polar or charged amino acid (His_{103}, Asp_{104}, Gln_{109}, or Pro_{117}) with a hydrophobic residue. Interestingly, each of the polar or charged residues in the prM signal sequence, other than Gln at the -2 and -4 positions with respect to the cleavage site, was targeted in one of the variants derived from VPQAQA RNA transfections. The reversions or second-site substitutions in the prM signal sequence were sufficient to almost fully restore growth properties in vertebrate and invertebrate (data not shown) cells of variants containing the VPQAQA mutation. One exception was the change of Thr_{107} to Ile, found in one variant, which probably required additional mutations outside the C and prM protein genes for viability. Variant V7(T_{107}→I) displayed delayed growth kinetics and a small-plaque phenotype on Vero cell monolayers in comparison to YFV 17D. No marked difference in plaque size was noted between a VPQAQA variant with a substitution at His_{103} or Pro_{117} in the prM signal sequence and strain 17D (data not shown); however, both variants were significantly attenuated in mouse neurovirulence. It thus appears that a slight increase in the hydrophobicity of the signal sequence restores the viability, albeit with altered neurovirulence properties, of YFV containing the VPQAQA mutation; the substitutions can involve single amino acids in the NH_2-terminal region or c-region of the signal sequence.

Surprisingly, none of the revertants or second-site mutants showed phenotypic reversion in the efficiency of the signal peptidase cleavage of prM in cell-free expression assays. However, we cannot exclude the possibility that the additional mutations in the viable progeny derived from the VPQAQA mutant RNA had only a subtle effect on the signal peptidase cleavage of prM in vivo which was not apparent in the in vitro assay. Alternatively, the more efficient signal peptidase cleavage at the C-prM junction when independent of prior cleavage of the C protein may not be lethal per se for viral replication. A consequence of the rapid signal peptidase cleavage of prM would be the production of a membrane-anchored form of the C protein as the predominant processing intermediate. This consequence would be deleterious for virus replication if the membrane-anchored C protein functioned poorly or not at all as a substrate for the viral protease. This situation has indeed been noted in expression studies with the MVE structural polyprotein region with or without a prM signal peptide mutation (24) analogous to the VPQAQA mutation described here for YFV (data not shown). Accordingly, the sequential order of proteolytic processing events between the C and prM proteins may have evolved to accommodate the substrate requirements of the NS2B-3 protease and maintain efficient cleavage of the C protein.

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