

Processing of Yellow Fever Virus Polyprotein: Role of Cellular Proteases in Maturation of the Structural Proteins

ANDRÈS RUIZ-LINARES,† ANNIE CAHOUR,‡ PHILIPPE DESPRÈS, MARC GIRARD,
AND MICHÈLE BOULOY*

Unit of Molecular Virology, Centre National de la Recherche Scientifique UA 545, Institut Pasteur, 75724 Paris Cédex 15, France

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The yellow fever virus (YFV) cDNA segment coding for the part of the precursor polyprotein generating the structural proteins C (capsid), prM (precursor to the membrane protein M), and E (envelope) was expressed *in vitro* by using the T7 promoter-polymerase transcription system coupled to translation in rabbit reticulocyte lysates. A polypeptide of the expected molecular weight was observed to accumulate in the assay and was processed into proteins C, prM, and E only when dog pancreas microsomal membranes were added to the translation system. Proteins prM and E were translocated inside the endoplasmic reticulum, where prM underwent glycosylation. Regions essential for translocation of these proteins were localized to the 18- and 15-amino-acid C-terminal hydrophobic regions of proteins C and prM, respectively. Translocation of protein prM appeared to be less efficient than that of protein E. Maturation of these proteins followed different kinetics, indicating that the prM signal is probably cleaved off more slowly. A polypeptide composed of proteins C and prM, similar to the NVx polypeptide described in yellow fever virus-infected cells, was also produced in the *in vitro* system in the presence of membranes. No mature protein M was detected, suggesting that the cleavage of prM to M is a late processing event mediated by a protease different from endoplasmic reticulum signalases.

Yellow fever virus (YFV) is the prototype of the *Flaviviridae* family which includes about 65 viruses, some of which are of major human health concern, such as yellow fever virus, dengue virus, and Japanese encephalitis virus (27, 48). The virus is small and enveloped, consisting of an icosahedral nucleocapsid containing the single-stranded RNA genome complexed with multiple copies of the basic capsid protein (C; molecular weight [MW], 13,000 to 16,000) surrounded by a host-derived membrane in which two viral proteins, the membrane protein M (MW, 8,000) derived from a glycosylated precursor prM (MW, 27,000) and the envelope protein E (MW, 51,000 to 59,000) (48), are inserted. The genomic RNA of positive polarity is the only mRNA produced during infection (14).

Sequence analysis of the genomes of several flaviviruses (6, 15, 16, 33, 34) has shown a similar organization. The viral RNA is approximately 11 kilobases long and has a single open reading frame expanding over more than 90% of the genome, making the flavivirus RNA among the longest of the eucaryotic messengers. By sequencing the N terminus of purified viral proteins from several flaviviruses, the gene order in the open reading frame has been determined to be C-prM-E-NS1-NS2A-NS2B-NS3-NS4a-NS4B-NS5 (1, 2, 4, 7, 32, 34, 37). It was concluded that all the viral proteins are derived from a high-MW precursor which must be cleaved to produce the individual structural and nonstructural proteins.

The proteases responsible for these cleavages are unknown, but it has been suggested that both cellular and viral proteases could be involved. According to the favored hypothesis, cellular signalases would be responsible for cleavage of the polyprotein precursor at hydrophobic regions which are located at the N and C termini of each of the

structural proteins and of the nonstructural protein NS1. These regions presumably act as signal peptides and stop transfer sequences and determine the translocation of proteins prM, E, and NS1 into the endoplasmic reticulum (ER). The cleavage of protein prM to generate mature protein M would be a late event performed by a Golgi protease recognizing the consensus sequence Arg-X-Arg/Lys-Arg present in several viral glycoproteins and in some hormone precursors (41). Alternatively, the presence of the tripeptide Cys-Trp-Cys in the prM amino acid sequence suggests that this protein might be an autoprotease, as this sequence is conserved at the active site of thiol proteases (33).

The processing of the polyprotein moiety comprising the nonstructural proteins NS2A to NS5 would require another enzyme(s) of cellular or viral origin which recognizes pairs of basic amino acids, either Arg-Arg or Lys-Arg, surrounded by short-side-chain amino acids. These cleavages would occur in the cytosolic phase, generating the nonstructural proteins, some of which could play a role as viral proteases, capping enzymes, or replicases (33, 40, 41).

The strategy of flavivirus protein synthesis is still poorly understood because of a lack of experimental data. In infected cells the complete polyprotein precursor has never been detected, although when infection was carried out under special conditions, some high-MW polypeptides were observed (9, 11, 30). Furthermore, *in vitro* translation of the genomic RNA in rabbit reticulocyte lysate (RRL) gave a complex pattern of polypeptides in which no specific viral products could be identified (28, 47). However, discrete bands corresponding to proteins E and C could be observed when tick-borne encephalitis virus RNA was translated in Krebs II cell extracts (22, 42, 43). Interestingly, the production of these proteins was dependent on the membrane fraction of the extract, indicating that cellular proteases included in this fraction probably were responsible for the maturation of the polyprotein.

In the present work, we have used an *in vitro* translation system to study the role of the cellular proteases involved in

* Corresponding author.

† Present address: Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom.

‡ Present address: Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

the cleavage of the YFV polyprotein precursor generating proteins C, prM, and E. In vitro transcripts were synthesized by using the T7 promoter-polymerase system and subsequently translated in RRL in the presence or absence of dog pancreatic microsomal membranes. The transcript coding for the structural proteins C-prM-E was translated into a polyprotein precursor, which was efficiently cleaved into polypeptides corresponding to authentic proteins C, prM, and E in the presence of membranes. We also detected an incompletely cleaved precursor composed of proteins C and prM. Progressive deletions of the N-terminal region of the polyprotein permitted us to localize the regions responsible for the translocation of proteins prM and E into membrane vesicles. We also showed that the putative N-type glycosylation sites of protein prM were efficiently recognized, whereas those of protein E were not. This confirms previous observations indicating that protein E from this specific strain of YFV is not glycosylated (17; P. Desprès et al., unpublished results).

MATERIALS AND METHODS

Materials. All restriction endonucleases and DNA-modifying enzymes, endoglycosidase H, phenylmethylsulfonyl fluoride, and ribo- and deoxyribonucleotides were obtained from Boehringer Mannheim Biochemicals. Vector pGEM4, T7 RNA polymerase, RNase inhibitors, RQ1 DNase, RRL, dog pancreatic microsomal membranes, and unlabeled amino acids were purchased from Promega Biotec. Protein A-Sepharose was purchased from Pharmacia, Inc. Proteinase K was obtained from Sigma Chemical Co. [³⁵S]methionine (800 Ci/mmol) was purchased from Amersham Corp. All oligodeoxyribonucleotides used for sequencing were synthesized at the Institut Pasteur.

Construction of YFV in vitro expression plasmids. Plasmid pGX.1S, containing the complete region coding for the structural proteins and the first one-fourth of protein NS1, was obtained by subcloning the *AvaI*-*Bam*HI fragment derived from the YFV cDNA insert of plasmid pAP51 (12) into the *AvaI*-*Bam*HI sites of plasmid pGYF5' (Fig. 1A), which contains a 1,000-base-pair insert starting with the first nucleotide of the YFV genomic sequence located 5 nucleotides downstream of the T7 promoter transcription start point (35). Plasmid pGX.1S/prM, deleted from part of the prM region, was obtained by successive treatments of pGX.1S with *Nde*I, *Ava*I, and Klenow fragment as indicated in Fig. 1A.

Figure 1B shows the construction of plasmid pGX.4, which is derived from pGYF5' and has conserved the YFV5' noncoding region, including the initiating ATG, followed by the polylinker region of pGem4.

Several restriction fragments isolated from pGX.1S and representing various parts of the YFV structural proteins were subcloned into pGX.4 digested with *Acc*I, *Sal*I, or *Hinc*II (depending on the phase of the insert), Klenow treated, and digested with *Bam*HI in order to orient the insert (Fig. 1C).

General DNA methods. Restriction endonucleases, Klenow enzyme, and ligase were used as recommended by the manufacturer; specific deoxynucleotide triphosphates were omitted when partial Klenow filling in was desired. Treatment with S1 exonuclease was performed in a total volume of 50 μ l containing up to 10 μ g of plasmid DNA, 250 mM NaCl, 1 mM zinc acetate, and 50 mg of bovine serum albumin per ml in the presence of 5 U of S1 nuclease. The reaction mixture was incubated for 30 min at 25°C, phenol

extracted, and ethanol precipitated. Samples were treated with Klenow before ligation in order to repair overhangs. Restoration of the reading frame was verified in each construct by direct sequencing of the plasmid (8) by using an oligodeoxynucleotide complementary to the sequence of T7 promoter, except for construct pGX.1S/prM, for which an oligodeoxynucleotide hybridizing at the 3' end of the prM coding region (position 810 to 794) was used. HB 101 bacteria were rendered competent and transformed with the plasmids. Other molecular biological manipulations were performed by using standard protocols (25).

In vitro transcription and analysis of transcripts. Linearized plasmids (1 μ g) were transcribed in 50 μ l of a mixture containing 20 mM KHPO₄ (pH 7.5), 10 mM dithiothreitol, 2 mM MgCl₂, 4 mM spermidine, 50 μ M of each ribonucleotide triphosphate, RNasin (1 U/ μ l), and T7 RNA polymerase (0.5 U/ μ l). The reaction mixture was incubated for 1 h at 37°C, and the DNA template was eliminated by treatment with 2 U of RQ1 DNase for 15 min at 37°C. The samples were phenol-chloroform extracted and ethanol precipitated twice with 2 M ammonium acetate. mRNAs thus obtained were finally taken up in sterile water, and their concentrations were determined by measuring the optical density at 260 nm.

In vitro translation and analysis of translation products. Translation was performed in a rabbit reticulocyte lysate system. The standard reaction mixture (12 μ l) contained 60% of the commercial preparation of RRL, 20 μ M of each amino acid except methionine, 1 mCi of [³⁵S]methionine per ml (800 Ci/mmol), 1 mM magnesium acetate, 170 mM potassium acetate, and RNA. Incubation was carried out for 60 min at 30°C, and the radioactivity incorporated into hot trichloroacetic acid-precipitable material was estimated for 3- μ l samples by using the protocol recommended by the RRL manufacturer (Promega Biotec).

Translation in the presence of microsomal membranes was carried out by the addition of 1 μ l of membranes into the reaction mixture described above.

Translation products were analyzed on 14% polyacrylamide gels after heat denaturation in the presence of 2% sodium dodecyl sulfate and 1% β -mercaptoethanol (21). When immunoprecipitated, the translation products were diluted to 100 μ l in a buffer containing 1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris hydrochloride, pH 7.5, and 1 mM EDTA and were incubated overnight at 4°C in the presence of antibodies (up to 5 μ l) and protein A-Sepharose. After extensive washing, the immunoprecipitates were eluted from protein A-Sepharose by boiling them in Laemmli denaturation buffer.

Enzymatic treatment of translation products. Proteinase K treatment was performed in a volume of 10 μ l containing 6 μ l of translation mix and proteinase at a concentration of 0.2 mg/ml. After incubation on ice, phenylmethylsulfonyl fluoride freshly dissolved in isopropanol was added at a concentration of 1 to 2 mg/ml. After a further 5-min incubation on ice, 30 μ l of sodium dodecyl sulfate-Laemmli buffer was added and the samples were boiled immediately for 5 min and loaded onto gels (26).

Endoglycosidase H treatment was carried out with 8- μ l samples of the translation mix, which were diluted into 100 μ l of 0.1 M sodium citrate (pH 5.5) containing 0.1% sodium dodecyl sulfate. After denaturation at 100°C for 2 min and cooling, phenylmethylsulfonyl fluoride at a final concentration of 1 to 2 mg/ml and 5 mU of endoglycosidase H was added and the samples were incubated overnight at 37°C.

Reactions were stopped by adding 1 ml of 20% trichloroacetic acid. After 30 min on ice, the precipitates were

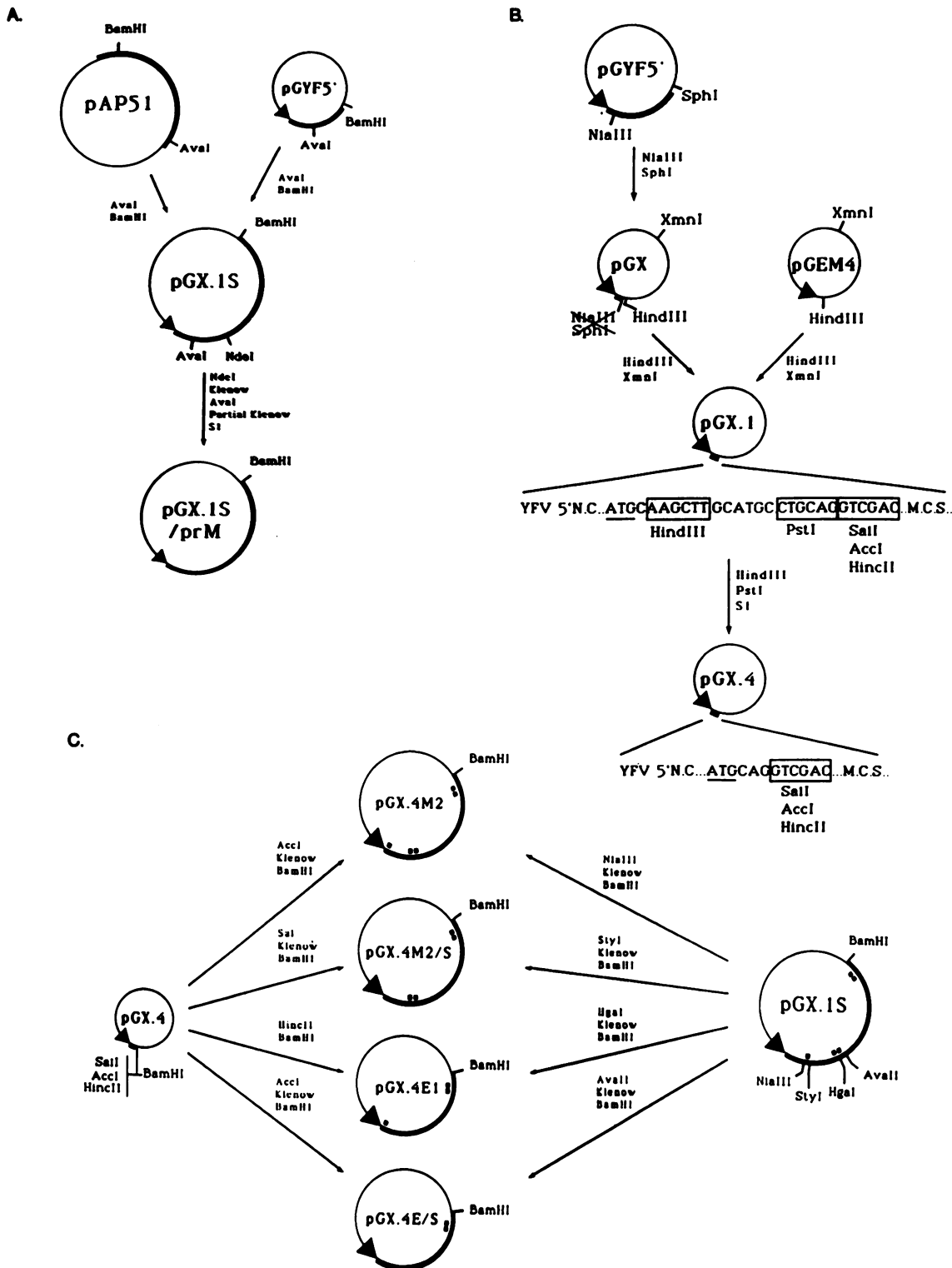


FIG. 1. Schematic diagram of the strategy used to construct YFV in vitro expression plasmids. (A) Reconstruction of the complete structural region of YFV cDNA from overlapping plasmids pAP51 and pGYF5'. Plasmid pGX.1S includes the first 2,725 bases of the YFV genome coding for proteins C, prM, E, and the first 92 amino acids of NS1. An internal deletion of the N-terminal half of protein prM was introduced in pGX.1S to obtain pGX.1S/prM by using appropriate restriction sites. YFV sequences are shown as bold lines, and vector sequences are shown as thin lines (PBR327 in plasmid pAP51 and pGEM in pGYF5'). The T7 promoter is indicated by an arrowhead. (B) Construction of plasmids containing the YFV 5' noncoding (YFV 5'NC) region downstream of the T7 promoter. The multiple cloning site (MCS) of vector pGEM4 was placed downstream of the YFV 5'NC region and was subsequently modified with S1 nuclease to eliminate excess nucleotides between initiating ATG (underlined) and the recognition sites for the enzymes *Sall*, *AccI*, and *HincII*. This site was chosen because it permits cloning in all three reading frames. Nucleotide sequences at the YFV junction are given for pGX.1 and pGX.4. Relevant restriction sites are boxed. Other symbols are as in panel A. (C) Construction of plasmids designed to localize translocation signals of proteins prM and E. All plasmids were derived from pGX.1S by subcloning of appropriate fragments of the YFV cDNA insert into pGX.4 downstream of the YFV untranslated leader. Hydrophobic regions in the YFV structural proteins are indicated (■). Other symbols are as in panel A.

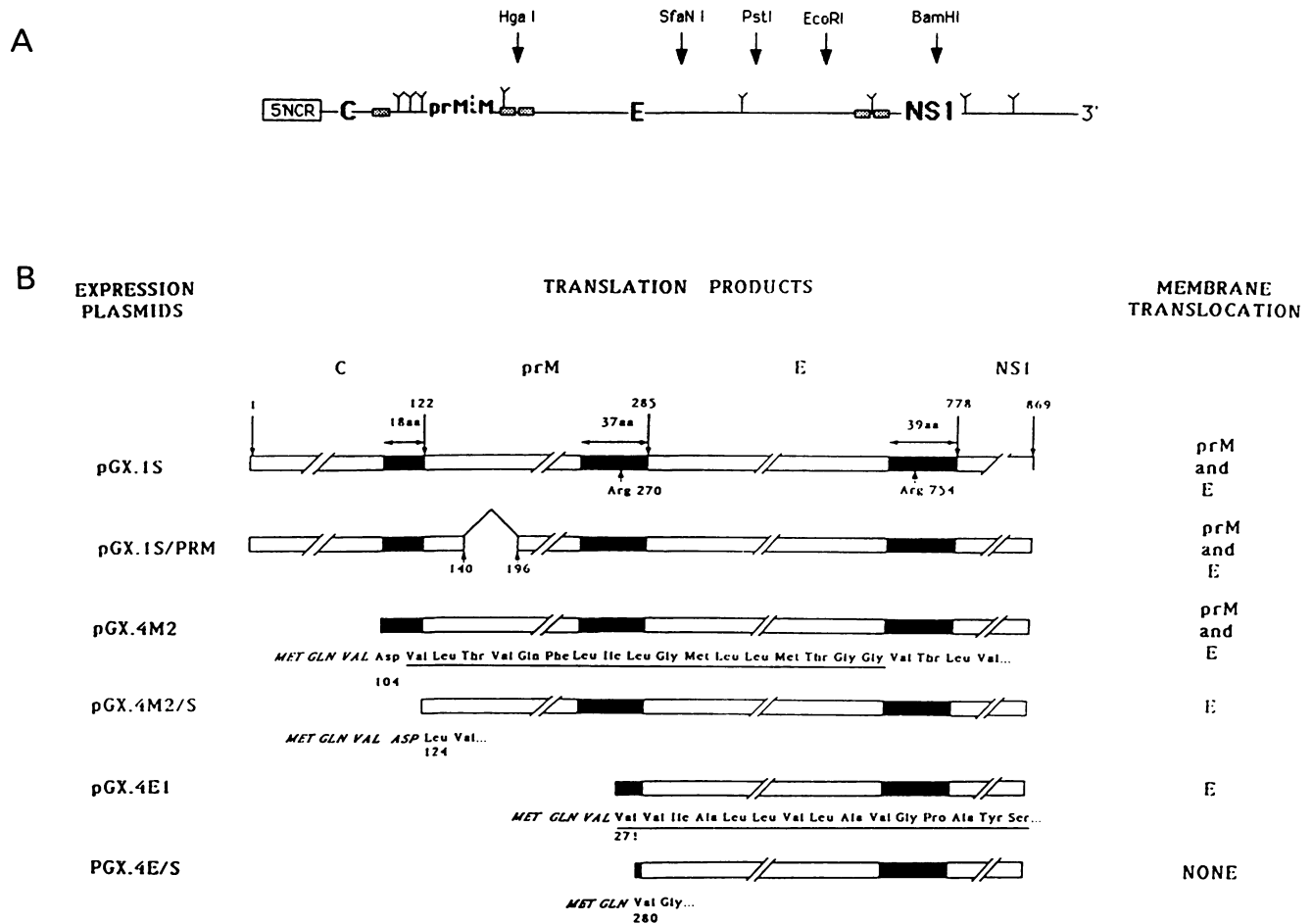


FIG. 2. Schematic diagrams of the proteins synthesized by the YFV in vitro expression plasmids. (A) Schematic representation of the YFV cDNA comprising the 5' noncoding region (5'NCR) and the region coding for proteins C, prM, E, and part of NS1. The positions of the restriction sites used to linearize the plasmids are indicated, as are the positions of the hydrophobic regions (■), delimiting the individual proteins. (B) Representation of the polyproteins encoded by different transcripts synthesized from *Bam*HI-linearized plasmids. Limits of the structural proteins are indicated, as is the position of the N-terminal residue of each protein. Protein NS1 is represented only by its first 90 amino acids, which are present in the precursor synthesized by these transcripts. Hydrophobic regions (■) of the precursor are indicated. The N terminus of each polypeptide encoded by the deleted constructs is indicated. Amino acids in italic type were derived from the construct and do not belong to YFV proteins. The complete sequences of the hydrophobic regions of proteins prM and E are indicated under the polypeptides encoded by pGX.4M2 and pGX.4E1, respectively. All numbers refer to amino acid positions on the complete YFV polyprotein. The proteins translocated in the presence of membranes are indicated on the right.

centrifuged and the pellets were washed twice with ethanol-ether (1:1), suspended in 40 μ l of Laemmli buffer, and loaded onto polyacrylamide gels.

RESULTS

YFV structural protein precursor has no autoproteolytic activity. Since it was suggested that protein prM might be an autoprotease, we first tested the stability of the polypeptide comprising the amino acid sequence of proteins C-prM-E. This polypeptide was synthesized in vitro in RRL programmed by synthetic mRNA transcripts. For this purpose we constructed a plasmid, pGX.1S, which contains the region coding for proteins C, prM, and E and for the first 90 amino acids of protein NS1 (Fig. 1 and 2). Plasmid pGX.1S was derived by recombination from plasmids pAP51 (12) and pGYF5' (35). Runoff transcripts obtained from plasmid pGX.1S linearized at the *Bam*HI site were translated in RRL, and samples were withdrawn after 15, 30, 60, 120, and

180 min of incubation and analyzed on a polyacrylamide gel (Fig. 3A). A polypeptide of 90,000 MW, the MW expected for a C-prM-E-NS1 precursor, was synthesized with no evidence for processing to lower-MW products. This indicated that under our experimental conditions, no autoproteolytic activity was detected in the structural protein precursor. However, it cannot be excluded that the prM protein possesses a protease activity when glycosylated.

Induction of processing of the YFV structural protein precursor by microsomal membranes. To test the hypothesis that the processing leading to the formation of proteins C, prM, and E is due to cellular signalases, we supplemented the RRL with dog pancreatic microsomal membranes. Such a system has proved to be extremely useful for studying the topogenic signals for the transport of a number of cellular and viral proteins into the ER (3, 18, 19).

Transcripts of increasing length were synthesized from plasmid pGX.1S linearized at the *Sfa*NI, *Pst*I, and *Eco*RI

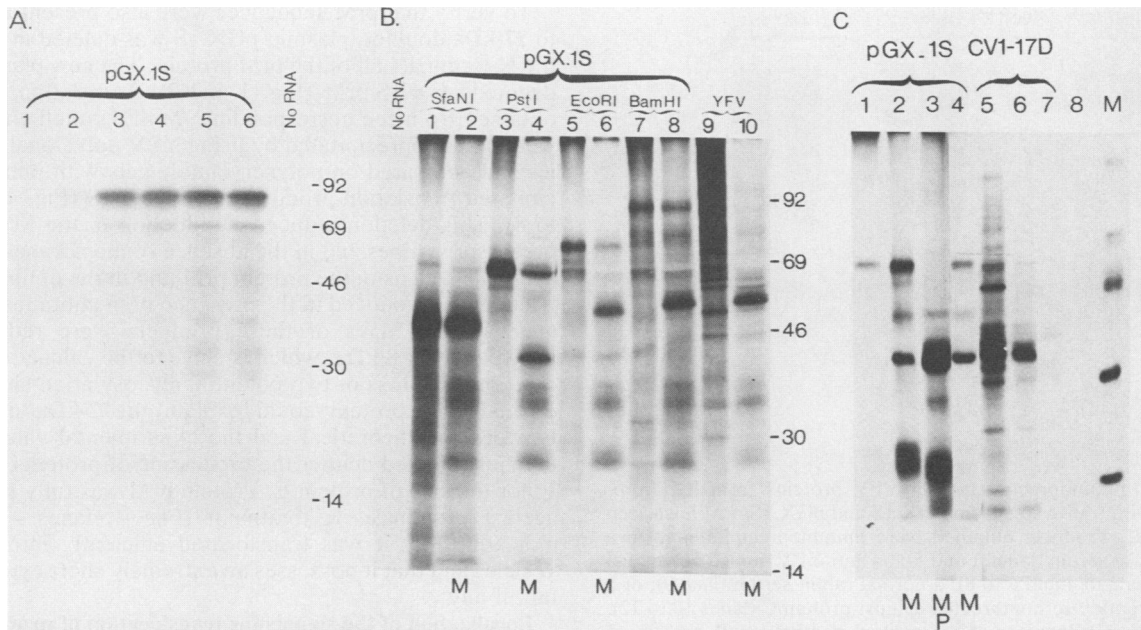


FIG. 3. Analysis of the translation products synthesized in RRL programmed with pGX.1S transcripts. (A) *Bam*HI runoff transcripts coding for a polypeptide ending at position 869 of the YFV polyprotein (Fig. 1C and 2) were translated in the absence of microsomes. Samples were collected after 0, 15, 30, 60, 120, and 180 min (lanes 1 to 6, respectively). MWs (in thousands) are indicated at the right. (B) pGX.1S transcripts of increasing size were synthesized from pGX.1S templates linearized at the *Sfa*NI, *Pst*I, *Eco*RI, and *Bam*HI sites. These transcripts encode polypeptides ending, respectively, at positions 340, 674, 717, and 769 of the YFV polyprotein (Fig. 2). Translation products synthesized from these transcripts in the absence or presence of membranes were compared with those obtained with the genomic RNA extracted from YFV virions. Products loaded on each lane are indicated. M, translations carried out in the presence of microsomal membranes. MWs (in thousands) are indicated at the right. (C) Immunoprecipitation of YFV proteins synthesized in vitro or in YFV-infected cells. Products obtained by in vitro translation of pGX.1S *Bam*HI transcripts were immunoprecipitated with a mouse polyclonal ascitic fluid directed against infectious viral particles (lanes 1, 2, and 3), a mouse monoclonal antibody (5H3) specific for protein E (lane 4), or a normal mouse serum (lane 8). Extracts of monkey kidney CV-1 cells infected with YFV were immunoprecipitated with YFV polyclonal antibodies (lane 5), anti-E monoclonal antibody (lane 6), or a normal mouse serum (lane 7). Where indicated, in vitro translations were performed in the presence of membrane (M [lanes 2, 3, and 4]) and submitted to proteinase K treatment (P) prior to immunoprecipitation. Lane M, Molecular size markers.

restriction sites within the region coding for protein E or at the *Bam*HI site within the region coding for protein NS1 (Fig. 2A). In the absence of membranes, the *Sfa*NI, *Pst*I, *Eco*RI, or *Bam*HI transcripts induced the synthesis of a polypeptide of the MW predicted from the coding capacity of the corresponding mRNAs (Fig. 3B, lanes 1, 3, 5, and 7). With regard to the YFV genomic RNA translation products (lane 9), the pattern of bands was extremely complex, thus making difficult the identification of any viral protein and confirming previous reports on the in vitro translation of other flavivirus genomic RNAs (28, 47).

(i) **Description of the products.** When membranes were included in the translation reaction (Fig. 3B, lanes 2, 4, 6, 8, and 10), the polypeptide precursor was clearly processed. Every transcript led to the synthesis of at least four polypeptides of 15, 27, 34, and 37 kilodaltons (kDa), respectively. On the basis of their molecular masses, the 15- and 27-kDa polypeptides are likely to be proteins C and prM, respectively. In many experiments the protein C band was difficult to detect unless the gel was exposed for a very long time. As will be shown below, the 34- to 37-kDa doublet corresponds to a polypeptide comprising proteins C and prM. In addition, a fifth band was observed when the *Pst*I, *Eco*RI, and *Bam*HI transcripts were translated. This polypeptide varied in size from approximately 40 kDa in the *Pst*I transcript-derived products (lane 4) to 54 kDa in the translation products from the *Bam*HI transcript (lane 8) or from genomic YFV RNA

(lane 10). As the N terminus of protein E corresponds to amino acid 285 in the polyprotein (32, 33), it could be deduced that the region of the mature protein E expressed from the *Sfa*NI, *Pst*I, and *Eco*RI runoff transcripts represents 55, 389, and 482 amino acids, respectively, while the *Bam*HI transcript contains the entire sequence of the E protein (Fig. 2A). Therefore, on the basis of the apparent MWs of the products, these results strongly suggest that in the presence of membranes, the precursor was processed to generate protein E. However, definite identification of the E protein was performed by immunoprecipitations.

(ii) **Identification of the envelope protein.** Two envelope-specific antibodies were used: a polyclonal ascitic fluid which reacted with most of the structural proteins and several nonstructural proteins and monoclonal antibody 5H3, which reacted with protein E (36). All the polypeptides synthesized from the *Bam*HI runoff transcripts were precipitated by the polyclonal antibodies (Fig. 3C; compare lanes 1 and 2 with lanes 7 and 8), but only the 54-kDa protein and the unprocessed precursor were recognized by monoclonal antibody 5H3 (Fig. 3C, lane 4). Other monoclonal antibodies specific for the envelope protein (kindly provided by A. Barrett and J. Schlesinger) were also reactive. As a control, [³⁵S]methionine-labeled proteins from YFV-infected CV1 cells were immunoprecipitated with either the polyclonal serum (lane 5) or the monoclonal antibody 5H3 (lane 6). As expected, the E proteins synthesized in vitro and in infected

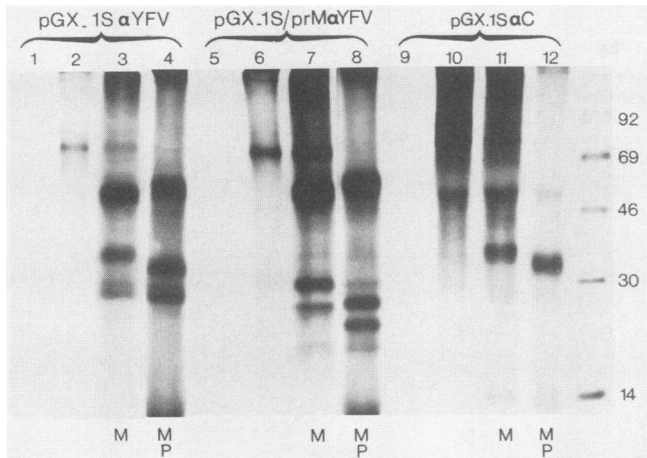


FIG. 4. Immunoprecipitation of YFV proteins from RRL programmed with transcripts from pGX.1S and pGX.1S/prM linearized with *Bam*HI. Products obtained were immunoprecipitated with a normal mouse serum (lanes 1 and 5); an anti-YFV mouse polyclonal serum (lanes 2 to 4 and 6 to 8); a normal rabbit serum (lane 9); or a rabbit antipeptide serum directed against protein C (lanes 10 to 12). M, Translation in presence of microsomal membranes; P, proteinase K treatment. Protein C was clearly visible only after a long exposure time. The 54-kDa band obtained in lanes 10 and 11 does not correspond to protein E, as it was observed in translation mixes performed in the absence of membranes (lane 10) and was proteinase K sensitive (lane 12); it represents an artifact of precipitation with the antipeptide antibody. MWs (in thousands) are indicated at the right.

cells comigrated. To confirm that the E protein was translocated into the ER, we showed that in the presence of membranes, the polypeptide was protected from degradation by proteinase K (lane 3) and became sensitive to the protease in the presence of Triton X-100 (not shown). Thus, these experiments demonstrate that generation of protein E occurred in the presence of membranes. The fact that the apparent MW of the protein was not affected by treatment with proteinase K indicated that its C terminus was not exposed on the cytoplasmic side of the membrane or that its exposure was limited to a few amino acids, in agreement with the model presented by Mandl et al. (23, 24).

(iii) **A C-prM precursor is synthesized and partially processed in vitro.** We next attempted to identify the 34- to 37-kDa polypeptide as the C-prM precursor. The presence of protein C sequences in this doublet was demonstrated by immunoprecipitation using a rabbit protein C-specific immune serum raised against a synthetic peptide corresponding to protein C amino acids 20 to 40. The serum recognized the unprocessed polyprotein, the 34- to 37-kDa polypeptide, and the protein C band, which appeared as a faint band on this autoradiogram (Fig. 4, lane 11). In many experiments, protein C was difficult to detect; it might be unstable, and cleavage of the C-prM precursor was a slow process (see Fig. 7 and below). The 27-kDa protein was not immunoprecipitated by either an anti-C immune serum or by the anti-E antibodies. It must therefore represent the prM protein moiety. One can observe in this gel, in the absence or presence of membranes, a non-specific band comigrating with protein E which was considered an artifact. The fact that the C-prM precursor migrated as a doublet suggests that the prM region underwent various degrees of glycosylation, a phenomenon frequently observed in *in vitro* translation systems (31, 38).

To verify that prM sequences were also present in the 34- to 37-kDa doublet, plasmid pGX.1S was deleted in frame of the N-terminal half of the prM protein. The new plasmid was denoted pGX.1S/prM (Fig. 1 and 2). Translation products obtained from the corresponding *Bam*HI runoff transcripts were immunoprecipitated by using YFV polyclonal antibodies and compared on polyacrylamide gels with immunoprecipitated translation products from pGX.1S (Fig. 4, lanes 1 to 8). This deletion induced a reduction in the MW of the precursor synthesized in the absence of membranes (lanes 2 and 6) as well as in the protein prM and in the doublet band, which were produced in the presence of membranes (lanes 3 and 7). The MWs of these products were reduced by approximately 8 kDa, which is close to the calculated 6-kDa deletion. The loss of two potential glycosylation sites in the deleted prM protein could explain the 2-kDa difference between the theoretical and the experimental values. This deletion affected neither the processing of proteins prM and E nor the size of protein E. Protein prM was fully protected against proteinase K treatment (Fig. 4, lanes 4 and 8), indicating that it was translocated efficiently into the ER vesicles and that it possesses an extremely short cytoplasmic tail, if any.

Localization of the signals for translocation of proteins prM and E. Experiments were next designed to localize the regions in the polypeptide precursor responsible for the translocation of proteins prM and E inside the membrane vesicles.

Computer analysis of the YFV polyprotein (20) identified three hydrophobic regions localized between each of the structural proteins (Fig. 2). The first one is located between proteins C and prM and spans amino acids 105 to 121 of the precursor, the second one is present between proteins prM and E at amino acids 250 to 287, and the third one is located at the C terminus of protein E at amino acids 740 to 778. The last two hydrophobic regions are in fact divided into two peaks spaced by a single positively charged residue (Arg), suggesting that they would act as a stop transfer signal and signal peptide, respectively. More precisely, the hydrophobic region at the end of protein C would be the signal for translocation of protein prM, and its stop transfer signal would correspond to the first half of the second hydrophobic region (amino acids 250 to 270). The second half of this hydrophobic domain (amino acids 271 to 285) would direct the translocation of protein E. In the third hydrophobic region, also divided into two peaks, the first domain would serve as a stop transfer signal for protein E and the second would serve as a signal peptide for protein NS1. According to this model, proteins prM and E would be type I membrane proteins, with their N termini exposed on the exoplasmic side of the membrane and their hydrophobic C termini embedded in the lipid bilayer. The nonstructural protein NS1 would also be translocated, in accordance with the fact that it is found in a glycosylated form which is either soluble or associated with the cell surface of the infected cell (5, 39).

As no C-terminal sequence data are available for any of the YFV proteins, it is not known whether small intergenic peptides are produced by cleavages at the C termini of proteins C and prM during the maturation of the structural proteins. Such cleavages could be necessary to expose the N terminus of the hydrophobic regions preceding proteins prM and E. Alternatively, these hydrophobic regions could function as internal signal sequences, as has been found for polytopic membrane proteins (18, 29). In the latter case, the C termini of the structural proteins would be determined by signalase cleavages at the interior of the ER and thus could

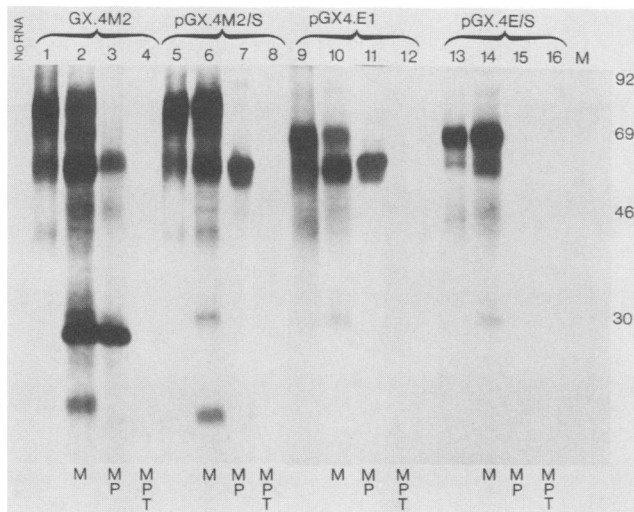


FIG. 5. Localization of regions essential for the translocation of proteins prM and E. Transcripts obtained from templates linearized at the *Bam*HI site were translated in the presence or absence of membranes (M) and submitted to a proteinase K treatment (P) in the presence or absence of Triton X-100 (T). Details of the polypeptides encoded by each transcript are given in Fig. 2. MWs (in thousands) are indicated at the right.

coincide with the N termini of proteins prM and E. However, this also implies that the C protein would be associated with membranes.

To define the role played by the hydrophobic regions of the precursor as signals for translocation of proteins prM and E, several plasmids were constructed.

Plasmid pGX4 was derived from plasmid pGYF5' by deletion of the YFV coding region and insertion downstream of the initiating ATG codon of the multiple cloning site from plasmid pGem4 (Fig. 1B). This plasmid produces *in vitro* T7 transcripts containing the 5' noncoding region of YFV. The initiation codon was conserved and followed by the multiple cloning site, which allowed the cloning in phase and efficient *in vitro* expression of different open reading frames (35). Different fragments of cDNA coding for various YFV structural proteins were then subcloned into plasmid pGX4 in order to produce plasmids with N-terminal deletions in the polyprotein precursor (Fig. 1C and 2). Four plasmids were thus constructed. pGX.4M2 coded for a polypeptide beginning with the hydrophobic region from the C terminus of protein C, and plasmid pGX.4M2/S coded for a polypeptide lacking this hydrophobic region but beginning at the third amino acid of protein prM. Plasmid pGX.4E1 expressed the region coding for protein E preceded by the second hydrophobic peak located after arginine 270 and present at the C terminus of protein prM. The last construct, pGX.4E/S, coded for a polypeptide beginning 5 amino acids upstream from the N terminus of the mature E protein.

The polyprotein expressed from pGX4M2 transcripts was processed, and proteins prM and E were efficiently translocated (Fig. 5, lanes 1 to 4), indicating that the first 103 amino acids of the capsid protein are unnecessary for translocation. Furthermore, the 37- to 34-kDa doublet disappeared, as would be expected for the C-prM precursor.

The sequence coding for the hydrophobic region at the C terminus of protein C was deleted in plasmid pGX.4M2/S. Thus the resulting transcript expressed a polyprotein, the N terminus of which corresponded to the third amino acid of

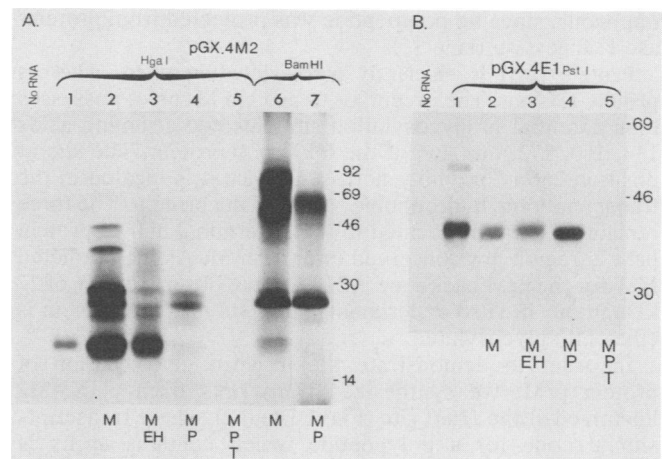


FIG. 6. *In vitro* glycosylation of proteins prM and E. Translation products were submitted to proteinase K (P) or endoglycosidase H (EH) treatment as indicated. M, Microsomal membranes; T, Triton X-100. (A) Transcripts obtained from pGX.4M2 linearized at the *Hga*I or *Bam*HI site (position 270 or 869 of the YFV polyprotein [Fig. 2]). (B) Transcripts obtained from pGX.4E1 linearized at the *Pst*I site (position 674 of the YFV polyprotein [Fig. 2]). MWs (in thousands) are indicated at the right.

protein prM. In this case, protein E was translocated but protein prM was not. Indeed, protein E was resistant to protease treatment, but the 16-kDa polypeptide which must represent the unglycosylated form of protein prM was not (Fig. 5, lanes 5 to 8). This result indicates that the region essential for protein prM translocation is located within the last 18 amino acids of protein C and that proteins prM and E bear independent translocation signals.

Deletion of most of the protein prM, with the exception of its C-terminal hydrophobic region (plasmid pGX.4E), did not alter the translocation of protein E (Fig. 5, lanes 9 to 12), but the absence of this hydrophobic zone in plasmid pGX.4E/S prevented the translocation of the protein (Fig. 5, lanes 13 to 16). This suggests that the last 15 amino acids in protein prM are necessary for the translocation of protein E, which confirms the prediction based on the rule of von Heijne (44, 45) that this region exhibits all the characteristics of a signal peptide. This also confirms the results of our recent *in vivo* studies using a simian virus 40 expression vector which indicate that the region within amino acids 271 to 285 of the YFV polyprotein acts as a signal peptide for the translocation of the envelope protein as well as for the heterologous poliovirus VPO protein (13; P. Desprès et al., manuscript in preparation).

From the experiments carried out with transcripts from pGX.4E/S, it is not clear whether NS1 was translocated or not (Fig. 6, lanes 13 to 16). However, the polypeptide contains the putative signal sequence for its translocation, and if processing of protein NS1 had occurred in the presence of membranes, the apparent MW of the precursor would have been reduced by approximately 10 kDa, generating two polypeptides of 10 and 60 kDa, respectively. The 10-kDa polypeptide was not detected, possibly because of its small size. In addition, analysis of the processed polypeptides (lane 14) did reveal the presence of a band about 10 kDa smaller than the precursor. However, since a similar polypeptide was synthesized in the absence of membranes, an unambiguous conclusion could not be drawn. These experiments indicate merely that the translocation of protein E did

not occur, since no polypeptide was protected from proteinase K digestion (lane 15).

Protein prM is efficiently glycosylated in vitro, whereas protein E is not. The precursor to protein M, prM, possesses four potential N glycosylation sites located at amino acids 134, 150, 172, and 266 of the YFV polyprotein. The site at position 266 is probably not used, since it is located in the transmembrane hydrophobic region of the protein. The three remaining sites are located in the N-terminal half of protein prM, a region not conserved in protein M. As the predicted MW for the prM backbone is 21 kDa, the observed MW of 27 kDa in our in vitro experiments suggests that this protein is efficiently glycosylated.

In order to demonstrate the in vitro glycosylation of protein prM, we synthesized transcripts from pGX.4M2 linearized at the *Hga*I site (Fig. 1C and 2). These transcripts should code for a polypeptide which contains at its N terminus the signal for translocation of protein prM but has deleted at its C terminus the 15 amino acids which precede the sequence of the envelope protein. The encoded polypeptide should have a MW close to that of the native prM protein. The pGX.4M2/*Hga*I transcripts code for a polypeptide of 18 kDa that is partially transformed into a 27-kDa polypeptide in the presence of membranes (Fig. 6A).

This polypeptide is glycosylated, as evidenced by its sensitivity to endoglycosidase H treatment (lane 3). As expected, the glycosylated form of prM is protected from proteinase K digestion (lane 4) but is digested in the presence of detergent (lane 5). A similar 27-kDa polypeptide is also synthesized and processed in translation reactions carried out with longer *Bam*HI transcripts (lanes 6 and 7), indicating that the C terminus of prM is in close proximity to the *Hga*I site.

Protein E possesses two potential N-linked glycosylation sites at asparagine residues 594 and 755 of the YFV polyprotein. As in the case of protein prM, the second site is thought to be nonfunctional because it is located in the C-terminal hydrophobic region at the C terminus of the protein. To investigate whether asparagine 594 could be effectively recognized, in vitro transcripts were synthesized from plasmid pGX4.E1 linearized with *Pst*I. These transcripts should code for the N-terminal region of protein E containing asparagine 594 followed by 20 amino acids. An expected polypeptide of 40 kDa was synthesized upon translation (Fig. 6B, lane 1). In this size range it should be easy to detect small variations in MW due to the addition of polysaccharide residues. Translocation products obtained in the presence of membranes (Fig. 6B, lane 2) were treated with proteinase K (lanes 4 and 5) and endoglycosidase H (lane 3). The observed product did not change in MW after the addition of membranes or after endoglycosidase H treatment, indicating that no glycosylation had occurred. However, the polypeptide was efficiently translocated, as demonstrated by its resistance to proteinase K in the absence of Triton X-100. The lack of glycosylation of protein E is in agreement with previous observations (17) and with the fact that the only accessible glycosylation site in the protein is found in a weak context for glycosylation.

Kinetics of YFV structural protein synthesis. To study the events leading to the production of the individual C, prM, and E proteins, pGX.1S-*Bam*HI transcripts were translated in the presence of membranes, and the products synthesized after different times of incubation were analyzed (Fig. 7A).

A product with an apparent MW close to 30,000 was first observed, followed by the simultaneous appearance of the 55-kDa protein E and the 37- to 34-kDa C-prM precursor.

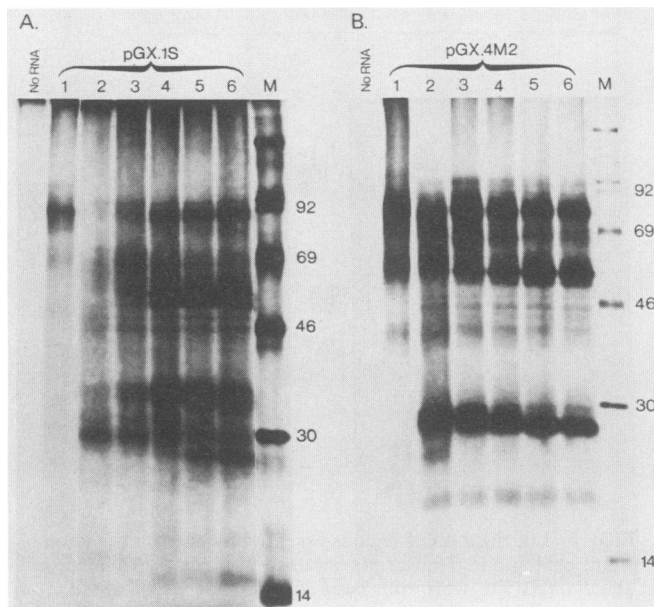


FIG. 7. Kinetics of processing of the YFV structure protein precursor. Transcripts from *Bam*HI-linearized pGX.1S (A) or pGX.4M2 (B) were translated in the absence (lane 1) or presence (lanes 2 to 6) of microsomal membranes. Samples of translation products obtained in the presence of membranes were collected after 15, 30, 45, 60, and 120 min (lanes 2, 3, 4, 5, and 6, respectively). Addition of membranes posttranslationally did not induce any protein processing (not shown). MWs (in thousands) are indicated at the right.

The latest event was the production of proteins prM (27 kDa) and C (15 kDa), presumably after cleavage of the C-prM precursor. The 30-kDa product possibly corresponds to an untranslocated C-prM precursor which after translocation increases in MW to 37 kDa due to glycosylation. It would thus seem that the prM signal peptide works with a moderate efficiency for translocation and is not cleaved cotranslationally but as a late event.

To confirm the kinetics of the processing of the C-prM precursor, transcripts derived from pGX.4M2 were analyzed in a similar kinetics experiment. The polypeptide encoded by this transcript begins with the 18-hydrophobic-amino-acid stretch found at the C terminus of the capsid protein (Fig. 2). When the products synthesized after various incubation periods were analyzed, protein E was already detectable after 15 min of incubation (Fig. 7B) and did not change in MW with time, while the protein prM was first synthesized as a 30-kDa product which was progressively processed into a 27-kDa product. This 3-kDa shift in MW most probably corresponds to the cleavage of the N-terminal region representing the signal peptide. In addition, a product of 18 kDa, probably corresponding to the unglycosylated (untranslocated) prM, was observed in many experiments.

Capsid protein is closely associated with the membrane of the ER in the C-prM precursor. Proteinase K treatment of pGX.1S translation products synthesized in the presence of membranes induced a reduction of approximately 3 kDa in the apparent MW of the C-prM precursor (Fig. 4, lanes 3, 4, 11, and 12). This implied that only the first 20 to 30 amino acids were exposed outside the membrane vesicles. Immunoprecipitation of the translation products of pGX.1S transcripts with the anti-C antibody provided confirmation of this observation (Fig. 4, lanes 9 to 12). The 30- to 31-kDa

doublet observed after proteinase K digestion was still recognized by the C-specific antibodies (Fig. 4, lanes 11 and 12). Since this antibody is directed against capsid protein amino acids 20 to 40, the region digested by proteinase K must not extend very much further than the first 20 amino acids. As the capsid protein is fairly hydrophobic from residue 42 onward, it is possible that the rest of the protein is closely associated with the membrane and thus is protected from proteinase K digestion.

Unfortunately, it was not possible to verify whether this partial resistance of protein C to proteinase K digestion also occurred when it was cleaved from the C-prM precursor, because protein C could only be detected clearly on gels after long exposure times. This problem was worse when immunoprecipitations were performed. For this reason and because of the poor resolving power of these gels for low-MW polypeptides, we could not determine whether the capsid protein was partially or totally degraded after proteinase K treatment.

DISCUSSION

We have established an *in vitro* transcription-translation system to study the processing of the YFV polyprotein and to define the role played by cellular proteases in the maturation of the viral structural proteins.

By using this approach, it was found that (i) production of proteins C, prM, and E was dependent on the addition of microsomal membranes to the translation system. Proteins prM and E were translocated inside the ER membrane, where protein prM, but not protein E, was glycosylated. The translocated proteins are totally resistant to proteinase K digestion, indicating that they do not have a domain located in the cytoplasmic side of the membrane but most likely are anchored in the membrane by the hydrophobic regions present at their C termini. Proteins prM and E are thus membrane proteins of the type I, with their N termini exposed on the exoplasmic side of the membrane and their C termini anchored in it.

(ii) Translocation of proteins prM and E inside the ER vesicles is dependent on the last 18 or 15 amino acids present at the carboxylic ends of proteins C and prM, respectively. These two signals function independently of each other; they are active when located in an N-terminal position from the protein they translocate, and the prM signal, at least, can also function as an internal translocation sequence.

(iii) The prM signal seems to be less efficient for translocation than the E signal, since a significant amount of prM is not translocated inside the ER membrane, whereas nearly all of the E protein is translocated. Furthermore, cleavage of the prM signal is not complete and takes place after translocation of the protein, as evidenced by the constant presence of a translocated C-prM precursor.

(iv) Protein C is not translocated inside the ER vesicles but remains very closely associated with the ER membrane by its C terminus, at least in the form of the translocated C-prM precursor. The existence of the translocated C-prM precursor suggests that previous exposure of the N terminus of the hydrophobic region preceding protein prM is not essential for its translocation. Thus, it would function as an internal signal sequence.

These results confirmed the schematic representation proposed by Coia et al. (10), which demonstrate the dependence of the processing of the YFV polyprotein on cellular proteases. The cellular protease involved must be of the signalase type, recognizing cleavage sites located after signals for

translocation at the N-terminal parts of prM and E proteins. Cleavage of prM to produce M was not effected in our system, suggesting that it is mediated by a protease present in the export vesicles of the cell. Cleavage of the C terminus of the capsid protein seems to be initially performed by the signalase that liberates the N terminus of prM. This would leave a capsid protein associated with membranes, the C terminus of which has to be cleaved a second time in order to make the capsid protein available for RNA encapsidation. It is possible that the second cleavage is effected by the viral protease responsible for the cleavages of the polyprotein precursor yielding the nonstructural proteins. This enzyme recognizes pairs of basic residues followed by a small-side-chain amino acid. Such a sequence is found just N terminal to the membrane-spanning domain of the capsid protein and is conserved in most flaviviruses (23). Maturation of the virus could thus be regulated by complex kinetics of cleavage, leading to isolated capsid, membrane, and envelope proteins.

Processing of the YFV polyprotein seems to be an interesting model of polytopic protein processing. Indeed, a series of translocation and stop transfer signals are found separating each structural protein and protein NS1. The structural proteins of the *Flaviviridae* seem to be unique in that the stop transfer and signal for translocation are located in a single hydrophobic region, separated only by a basic residue. In the present report we have delimited the regions essential for translocation of the prM and E proteins. Further studies are needed to show whether these regions are sufficient for translocation and whether they are signal recognition particle dependent, which would confirm their role as signal peptides (46). Further characterization of the regions acting as stop transfer signals is needed in order to relate the internal translocation signal to those found in type II membrane proteins. This seems particularly relevant in the case of the hydrophobic region separating proteins C and prM, as the C-prM precursor has an orientation relative to the ER membrane which is typical of type II proteins with a cytoplasmic N terminus and a luminal C terminus. Interestingly, the two potential signal peptides differ in their competence for translocation as well as in the kinetics with which they are cleaved off. Such a difference in signal efficiency could be important for the regulation of the viral life cycle, as it would regulate the rate of maturation of virus particles.

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