JOURNAL OF VIROLOGY, Nov. 1991, p. 6042–6050
0022-538X/91/116042-09$02.00/0

Vol. 65, No. 11

Processing of the Yellow Fever Virus Nonstructural Polyprotein: a Catalytically Active NS3 Proteinase Domain and NS2B Are Required for Cleavages at Dibasic Sites

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The vaccinia virus-T7 transient expression system was used to further examine the role of the NS3 proteinase in processing of the yellow fever (YF) virus nonstructural polyprotein in BHK cells. YF virus-specific polyproteins and cleavage products were identified by immunoprecipitation with region-specific antisera, by size, and by comparison with authentic YF virus polypeptides. A YF virus polyprotein initiating with a signal sequence derived from the E protein fused to the N terminus of NS2A and extending through the N-terminal 356 amino acids of NS5 exhibited processing at the 2A-2B, 2B-3, 3-4A, 4A-4B, and 4B-5 cleavage sites. Similar results were obtained with polyproteins whose N termini began within NS2A (position 110) or with NS2B. When the NS3 proteinase domain was inactivated by replacing the proposed catalytic Ser-138 with Ala, processing at all sites was abolished. The results suggest that an active NS3 proteinase domain is necessary for cleavage at the dibasic nonstructural cleavage sites and that cleavage at the proposed 4A-4B signalase site requires prior cleavage at the 4B-5 site. Cleavages were not observed with a polyprotein whose N terminus began with NS3, but cleavage at the 4B-5 site could be restored by supplying the NS2B protein in *trans*. Several experimental results suggested that *trans* cleavage at the 4B-5 site requires association of NS2B and the NS3 proteinase domain. Coexpression of different proteinases and catalytically inactive polyprotein substrates revealed that *trans* cleavage at the 2B-3 and 4B-5 sites was relatively efficient when compared with *trans* cleavage at the 2A-2B and 3-4A sites.

Yellow fever virus (YF), the prototype member of the family *Flaviviridae* (for reviews, see references 4, 7, 37, 47, 48), contains a single-stranded positive-sense RNA genome of 10,862 bases (35) which is capped but not polyadenylated. This RNA contains a single long open reading frame encoding a polyprotein of over 350 kDa (35). The gene order is 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′, where C, prM, and E denote structural proteins or their precursors and NS1 through NS5 indicate the nonstructural proteins. Mature flavivirus proteins are produced by cotranslational and posttranslational proteolytic processing using a combination of host proteinases and at least one viral proteinase (reviewed in references 7 and 36). Evidence suggests that the structural protein precursors and the N termini of NS1 and NS4B are generated by signalase in association with the membranes of the endoplasmic reticulum (9, 13, 26, 30, 38). Processing of the NS1-2A region probably involves host membranes, and the proteinase responsible for cleavage at the NS1-2A site (8, 18, 41) has not been identified. Downstream sequences in NS2A can influence cleavage at this site, and in some viruses, alternative cleavages within the NS2A region may occur (9, 13, 27). Cleavages generating the N termini of the nonstructural proteins NS2B, NS3, NS4A, and NS5 and the C terminus of the virion C protein (30, 42) follow double basic residues [consensus sequence G(A)RR ↓ S(G) for YF] (see reference 7 for a review), and although polyproteins have been identified in YF-infected cells, these processing events appear to occur rapidly and efficiently (9).

Molecular modelling studies and sequence comparisons have identified a serine proteinase domain within the N-terminal one-third of the 70-kDa NS3 protein (1, 16). Specifically, this region of NS3 contains homology to the trypsin family of serine proteinases and contains residues (His-53, Asp-77, and Ser-138 in YF) spatially equivalent to those found in the active site catalytic triad of this family of proteinases (1). Cell-free translation studies have demonstrated that polyproteins derived from YF, dengue virus type 2 (DEN2), or West Nile virus NS2A-NS3 regions containing the putative NS3 proteinase domain are capable of dilution-insensitive, site-specific cleavage to generate the N termini of NS2B and NS3 (11, 31, 45). At most, 181 (YF) or 184 (DEN2) residues of NS3 were required, and in addition, large deletions in DEN2 or DEN4 NS2B abolish cleavage at the 2A-2B and 2B-3 sites (14, 31). The importance of the proposed catalytic triad of the YF NS3 proteinase for these cleavages has been demonstrated by site-directed mutagenesis, and mutations which abolish or reduce cleavage activity in vitro have been shown to be deleterious for YF replication (7). Although experimental evidence is lacking, the presence of the consensus sequence at the cleavage sites generating the N termini of the nonstructural proteins NS4A and NS5 suggests that the NS3 proteinase domain is also involved in the processing at these sites. Using transient expression of YF polyproteins in BHK cells, we analyzed cleavage events in the YF nonstructural region and obtained evidence that a catalytically active NS3 proteinase domain is necessary, but not sufficient, for cleavages at all the nonstructural dibasic sites. In addition to the putative catalytic NS3 domain, NS2B is required for cleavage at least at the 4B-5 site, which can be processed in *trans* possibly by a complex consisting of NS2B and the NS3 proteinase domain.

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MATERIALS AND METHODS

Cells and viruses. YF 17D stocks were produced by infection of SW-13 cells as previously described (8). A vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase, vTF7-3 (15), was passaged in BHK-21 cells. Large-scale preparations of this virus were grown in BHK-21 cells and titered on BSC-40 cells. Growth of BHK-21 and BSC-40 cells has been described previously (8). BHK-21 clone 15 cells (BHK-21-15), used for transient expression assays, were obtained from Peter Mason and grown in Earle's minimal essential medium containing 7.5% fetal bovine serum.

T7 transcription plasmids. pET plasmids were provided by F. W. Studier, Brookhaven National Laboratories (43). pET-8c, also called pET-3d (43), is a transcription-translation vector with the T7 bacteriophage gene 10 promoter, leader, and terminator. These signals flank a convenient *Nco*I restriction site (CCATGG) such that the ATG codon is in a preferred context for efficient translation initiation in both *Escherichia coli* (43) and eukaryotic cells (21, 22). pTM3, obtained from B. Moss, National Institute of Allergy and Infectious Diseases, contains a T7 promoter and termination signal and the encephalomyocarditis virus 5′ untranslated region which facilitates cap-independent ribosome binding (29). In most cases, YF cDNAs were positioned immediately after the unique *Nco*I site in pET-8c or pTM3 which contains the ATG codon used for translation initiation. Under the transient expression conditions described below, similar results were obtained with either pET-8c or pTM3 derivatives. Plasmid constructions were obtained by using standard methods and reagents (40), and the structures were verified by digestion with appropriate restriction enzymes and sequence analysis. YF cDNA clones which have been successfully used to recover infectious YF RNA transcripts were utilized for these constructions (33–35). All subclones obtained from YF cDNA, amplified by using specific primers and the polymerase chain reaction (PCR) (39), were verified by sequence analysis.

Structure and nomenclature of YF cDNA constructs. A schematic of the polyprotein constructs utilized in this study is shown in Fig. 1, and a brief description of the important features of these constructs is given below. An abbreviated nomenclature is used to describe these plasmid constructs and the encoded polyproteins (Fig. 1). Constructs are designated by the N- or C-terminal boundaries of the polyprotein region they contain (without the NS prefix) with the amino acid residue numbers of truncated YF proteins (from the N terminus) indicated by subscripts. For example, the polyprotein construct containing the entire NS2B protein and the first 181 amino acids of NS3 is designated 2B-3_{181}. Constructs containing the NS3 Ser-138-to-Ala mutation, which abolishes in vitro cleavage activity (11), are indicated by an asterisk.

Construction of 2B-3_{181} and $2A_{110}$-3_{181} has been previously described (11). For construction of 3_{181}, YF cDNA from nucleotides (nt) 4571 to 5113 (from pYFM5.2 DNA [34]) was amplified by PCR to create a 5′-flanking *Nco*I site, adjacent UAA and UAG termination codons following NS3 residue 181, and a 3′ *Bgl*II site. PCR products were digested with *Nco*I and *Bgl*II, and the 550-bp fragment was ligated into pET-8c after digestion with *Nco*I and *Bam*HI. The 3-5_{356} construct was derived from 3_{181} by subcloning a restriction fragment from pYFM5.2 to extend the 3′ YF sequence from nt 5114 to nt 8704 (nucleotide positions refer to the YF cDNA sequence [34, 35]). Similar methods were used to extend the 3′ termini of the 2B-5_{356} and $2A_{110}$-5_{356} constructs to nt 8704. The 3-$4A_{92}$ and 3_{540}-5_{356} constructs were derived from 3-5_{356} by creating in-frame deletions in the YF cDNA sequences from nt 6717 to nt 8704 (inclusive) and nt 4571 to nt 6187, respectively.

A construct encoding NS2B was made by using PCR amplification to position a UAA termination codon immedi-

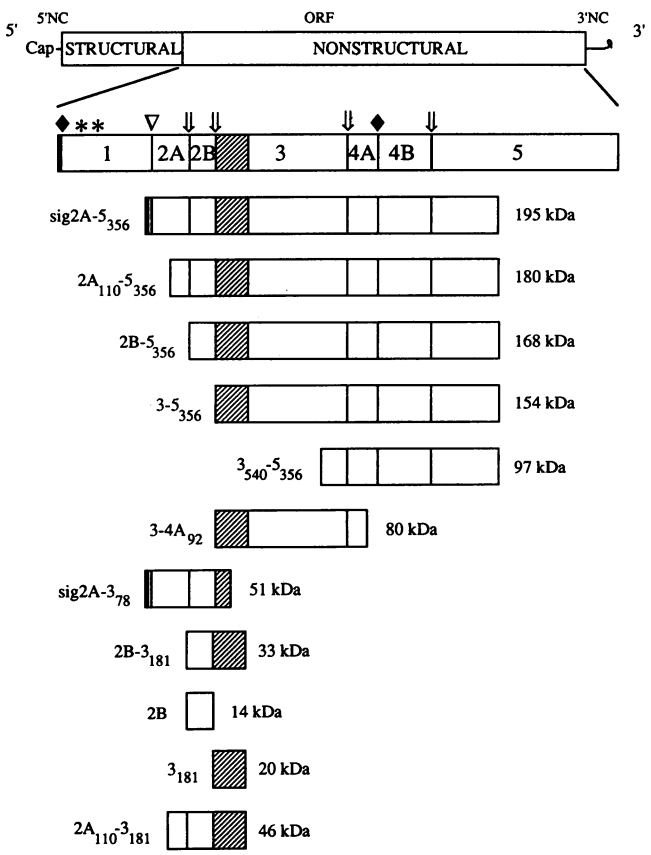

FIG. 1. Schematic of YF nonstructural polyproteins used in transient expression assays. General features of the YF17D genome including the 5′ cap, 5′ and 3′ noncoding (NC) regions, and the structural and nonstructural regions of the open reading frame (ORF) are shown. The nonstructural region from NS1 to NS5 is enlarged below, where 1, 2A, 2B, 3, 4A, 4B, and 5 indicate nonstructural proteins NS1, NS2B, NS3, NS4A, NS4B, and NS5, respectively. Asterisks indicate glycosylation sites of NS1. Symbols for cleavage sites are as follows: ◆, signalase cleavages; ▽, NS1-2A cleavage; ⇓, dibasic cleavage sites. All YF cDNA constructs shown are in pET-8c vectors except sig2A-3_{78}, which is in pTM3 (the nomenclature of these constructs is described in Materials and Methods). Hatched boxes indicate the proposed proteinase domain comprising the N-terminal one-third of NS3. Black boxes indicate a signal sequence, included as part of the constructs sig2A-5_{356} and sig2A-3_{78}. Some of the N or C termini contain non-YF sequences derived from other proteins or the cloning procedures: sig (23 amino acids derived from the C terminus of YF E, an Ala residue, and the first 12 residues of NS1); NS2B, 3_{181}, and 3-5_{356} (2 extra N-terminal amino acids, Met-Ala); sig2A-3_{78} (5 C-terminal non-YF residues); 3-$4A_{92}$ (9 C-terminal non-YF residues); and constructs ending at 5_{356} (13 C-terminal non-YF residues). Additional constructs not shown include sig2A-5_{356}*, 3-5_{356}*, 2B-3_{181}*, and $2A_{110}$-3_{181}*, which are identical to their homologs except for an NS3 Ser-138-to-Ala substitution. Predicted molecular masses of the polyproteins as deduced from the nucleotide sequences are shown to the right. For clarity, portions of the figure are not drawn exactly to scale.

ately following the C terminus of NS2B, and this was followed by a *Bam*HI restriction site. The PCR product was digested with *Sst*I (4334) and *Bam*HI and ligated with appropriate restriction fragments from 2B-3_{298} (derived from 2B-5_{356} by deletion of YF nt 5459 to 8704) to construct the full-length NS2B gene in pET-8c.

Polyproteins initiating with a signal sequence derived from the YF E gene fused to the NS2A gene, called sig2A, were derived by PCR amplification and several subcloning steps. These polyproteins begin with the C-terminal 23 residues of E (nt 2364 to nt 2452) and then the first 11 amino acids of NS1 (nt 2453 to nt 2485), an Ala residue (GCU; produced during subcloning), and then NS2A. sig2A-5_{356} was used to derive sig2A-3_{78} by deletion of the YF cDNA sequence from nt 4803 to nt 8704.

Derivatives of the constructs described above, containing the NS3 Ser-138-to-Ala mutation, were produced by substitution of an appropriate restriction fragment from 2B-3_{181}* which contains NS3 Ala-138 (11).

Transient expression assay. Nearly confluent monolayers of BHK-21-15 cells in 35-mm dishes (approximately 10^6 cells) were infected with vTF7-3 at a multiplicity of 10 PFU per cell. After 30 min, the inoculum was removed and 0.5 ml of minimal essential medium containing approximately 1 μg of plasmid DNA and 15 μg of lipofectin (Bethesda Research Laboratories) was added, and the cells were incubated for 2.5 h at 37°C. In experiments in which two or more plasmids were transfected, the total amount of DNA utilized was 1 μg. The transfection mixture was removed, and the medium was replaced with minimal essential medium containing 1/40th the normal concentration of methionine, 2% fetal bovine serum, and [^{35}S]methionine *trans* label (ICN) (20 to 40 μCi/ml) for 4 or 5 h. Cells were then washed with minimal essential medium, and lysates were prepared. [^{35}S]methionine-labeled YF proteins were produced in YF-infected BHK-21-15 cells by methods previously described for BHK-21 cells (8).

Immunoprecipitation and gel electrophoresis. As previously described (8), cell lysates were prepared with sodium dodecyl sulfate (SDS) or Triton X-100-containing lysis solutions, and reacted with antisera to *trpE*-YF fusion proteins or YF hyperimmune ascitic fluid, and immune complexes were collected by using *Staphylococcus aureus* Cowan strain I (Calbiochem).

Gel electrophoresis. Immunoprecipitates were solubilized and analyzed by SDS-polyacrylamide gel electrophoresis (23) and then by fluorographic detection of radiolabeled proteins (3, 24).

RESULTS

Expression constructs and method of analysis. In contrast to previous studies examining cleavage at the 2A-2B and 2B-3 sites, attempts to utilize cell-free translation to examine processing at the 3-4A, 4A-4B, and 4B-5 sites in the YF polyprotein were complicated by complex patterns of translation products, making it difficult to study site-specific cleavages (data not shown). As an alternative, we used a vaccinia virus recombinant which synthesizes T7 RNA polymerase (vTF7-3 [15]) to drive the expression of pET-8c/YF cDNA constructs which had been used to transfect BHK-21-15 cells. Figure 1 shows a schematic of the pET-8c/YF constructs generated for these studies (see Materials and Methods for details and nomenclature). YF-specific polyproteins and cleavage products were identified by immunoprecipitation with region-specific polyclonal antisera to YF NS2B, NS3, NS4B, or NS5 and comparison with size standards and authentic proteins from YF-infected cells (2, 8, 9, 32). Specific antisera are not yet available for YF NS2A and NS4A; hence, polyproteins or mature products containing these regions could not be identified directly.

Cleavage activity of sig2A-5_{356}. Attempts to engineer constructs encoding the entire YF nonstructural region starting with the signal sequence for NS1 in the C-terminal portion of E (13) through NS5 were unsuccessful because of problems propagating these plasmids in *E. coli* (see also reference 34). As an alternative, we constructed sig2A-5_{356} (Fig. 1). This construct encodes a polyprotein which begins with a signal sequence derived from the C terminus of E and then 11 N-terminal amino acid residues of NS1, an Ala residue, the entire NS2A, NS2B, NS3, NS4A, and NS4B proteins, and the first 356 residues of NS5. This polyprotein is predicted to have a membrane topology similar to that of the native viral polyprotein based on the current model for flavivirus polyprotein processing (reviewed in references 7 and 36).

Transfection with sig2A-5_{356} (Fig. 2) generated proteins whose sizes and immunoreactivity were consistent with their identification as NS2B, NS3, and NS4B. This implied that cleavage at all the dibasic sites had occurred as well as cleavage at the putative signalase site generating the N terminus of NS4B. It should be noted that the variable appearance of NS4B as a doublet in these experiments has been observed previously in YF-infected cells (8, 9); however, the structural basis for these two forms has not been established. Two NS3- and NS4B-specific polyproteins of 150 and 115 kDa were also produced. Several NS3-related proteins were produced, including a 70-kDa protein consistent in size with NS3 and an ~75-kDa species which comigrated with the stable NS3-4A species found in YF-infected cells (see Discussion). This heterogeneity of NS3-related species may result from alternative signalase or NS3 proteinase cleavages within the NS4A region. Many minor bands seen in these immunoprecipitation reactions are either host or vaccinia virus specific but appear to be enhanced where immune complexes are being formed, particularly with the NS3-specific antiserum. Additional NS2B-specific proteins included a minor 18-kDa species and a heterogeneous group of larger proteins of 30 to 33 kDa. Although the predicted molecular mass for 2A-2B is 39 kDa, the 30 to 33-kDa products may represent this species since NS2A (8, 41, 42, 46) and NS2A-containing polyproteins (9) have been observed to migrate faster than expected when analyzed by SDS-PAGE. Alternative cleavages, possibly within the NS2A region, might also account for the 30- to 33- and 18-kDa forms (9, 27, 28). Antiserum to NS5 showed weak reactivity with the 150-kDa protein and a protein of 40 kDa, which corresponds to the size predicted for 5_{356} (data not shown). This antiserum was not utilized in subsequent experiments, and appearance of the 27-kDa NS4B was used to monitor cleavage at the 4B-5 site.

Cleavages generated by $2A_{110}$-5_{356}, 2B-5_{356}, and 3-5_{356}. Figure 2 also shows results of transfection with $2A_{110}$-5_{356}, 2B-5_{356}, and 3-5_{356}, which contain successive N-terminal deletions relative to sig2A-5_{356} (Fig. 1 and Materials and Methods). Similar to the sig2A-5_{356} construct, $2A_{110}$-5_{356} and 2B-5_{356} produced 150- and 115-kDa NS3- and NS4B-specific polyproteins and several NS3-specific proteins between 70 and 100 kDa. Both constructs generated NS2B and NS4B. A minor amount of NS2B was sometimes observed in reactions using antiserum to NS4B, although this was not consistently observed. $2A_{110}$-5_{356} generated an NS2B-specific protein of 24 kDa corresponding in size to $2A_{110}$-2B (predicted molec-

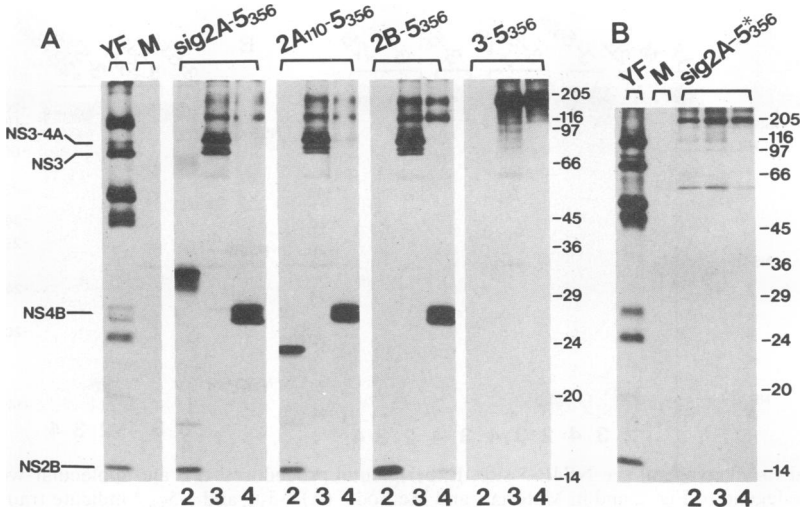

FIG. 2. Processing of a C-terminally nested set of YF polyprotein substrates. BHK-21-15 cells were infected with vTF7-3 and transfected with pET-8c/YF DNAs (Fig. 1) as described in Materials and Methods. Cells were labeled with [^{35}S]methionine for 4 h, and SDS-solubilized lysates were prepared and immunoprecipitated with antisera to YF nonstructural proteins. Antisera used for immunoprecipitation are indicated at the bottom, where 2, 3, and 4 indicate region-specific antisera to YF nonstructural proteins NS2B, NS3, and NS4B, respectively. Immunoprecipitates were analyzed by SDS-PAGE on 14% gels and then by fluorography. The positions of relevant YF proteins immunoprecipitated from YF-infected BHK-21-15 cells (YF) by using mouse hyperimmune ascitic fluid to YF are indicated. Samples from mock-transfected cells (lanes M), which had been infected with vTF7-3, were immunoprecipitated with a mixture of the three nonstructural protein antisera. Sizes of molecular weight standards ($\times 10^3$), as determined by Coomassie blue staining, are indicated at the right. (A) Transfection with polyprotein construct sig2A-5_{356}, 2A_{110}-5_{356}, or 3-5_{356}. (B) Transfection with polyprotein construct sig2A-5_{356}*, which contains the NS3 Ser-138-to-Ala mutation.

ular mass, 26.1 kDa). Surprisingly, although the 3-5_{356} construct contained the entire putative NS3 proteinase domain, only a 150-kDa polyprotein was observed whose size and immunoreactivity were consistent with uncleaved 3-5_{356}.

These results suggested that polyproteins containing nonstructural protein NS2A or NS2B through the N-terminal portion of NS5 are capable of undergoing cleavage reactions generating the N termini of NS2B, NS3, NS4A, NS4B, and NS5. The failure of 3-5_{356} to generate cleavage products suggested that NS2B was necessary for cleavage at the 3-4A and 4B-5 sites (see below). Alternatively, the 3-5_{356} construct contained two additional N-terminal amino acid residues (Met-Ala) which might be responsible for inhibiting cleavage at downstream sites.

Role of NS3 Ser-138 in processing of NS2-3-4-5 region. As mentioned previously, cell-free translation studies showed that the Ser-138-to-Ala mutation in the putative NS3 proteinase domain abolished cleavage at the 2A-2B and 2B-3 sites. The effect of this mutation on processing at these sites as well as the NS3-4A, NS4A-4B, and NS4B-5 cleavage sites was examined by transfecting cells with a derivative of sig2A-5_{356}, called sig2A-5_{356}*, which contained this substitution (Fig. 2B). In contrast to the results described above for sig2A-5_{356}, mature forms of NS2B, NS3, and NS4B were not observed in cells transfected with sig2A-5_{356}*. An uncleaved polyprotein of approximately 200 kDa which reacted with antisera to NS2B, NS3, and NS4B (predicted molecular mass of sig2A-5_{356}* is 194.1 kDa) was observed. A minor 115-kDa species which reacted with NS2B- and NS3-specific antisera (predicted molecular mass of sig2A2B3 is 110.5 kDa) and some additional high-molecular-weight NS3-related proteins were also observed. It is not clear whether these smaller products resulted from processing or degradation. Regardless, these results are consistent with the hypothesis that a functional NS3 proteinase domain is necessary for efficient cleavage at the 2B-3, 3-4A, and 4B-5 sites and perhaps indirectly for cleavage at the 4A-4B site (see below).

Evidence for NS2B participation in cleavage at the 4B-5 site. The observation that authentic processing occurred with 2B-5_{356} but not with 3-5_{356} suggested that NS2B is involved in proteolysis. The involvement of NS2B in cleavage at the 3-4A and 4B-5 sites was examined by cotransfecting constructs encoding the NS2B protein and the substrate 3-5_{356} or the catalytically inactive 3-5_{356}*. If NS2B and an active proteinase domain are both required for these cleavages, then coexpression of NS2B with 3-5_{356} but not 3-5_{356}* would be expected to yield NS3 and NS4B. Transfection with the NS2B construct yielded a 15-kDa NS2B-specific protein migrating similarly to NS2B produced in YF-infected BHK-21-15 cells (Fig. 3A). Transfection with either the 3-5_{356} or 3-5_{356}* construct alone yielded an NS3- and NS4B-specific polyprotein of 150 kDa and another NS3- and NS4B-specific polyprotein of slightly smaller molecular mass which may represent a host-mediated degradation product or result from preferred sites for premature translation termination. No mature NS4B was produced. Cotransfection of NS2B and 3-5_{356} generated an 83-kDa NS3-specific protein and the 27-kDa NS4B protein. These proteins were not observed after cotransfection of NS2B and 3-5_{356}*. These results indicated that both NS2B and an active proteinase domain are required for cleavage of the 4B-5 site and that NS2B can function when supplied in *trans*. Failure to observe the 70-kDa NS3 protein suggested that cleavage at the 3-4A site does not occur efficiently with NS2B supplied in *trans*.

Additional experiments were performed to determine whether cleavage at the 4B-5 site in 3-5_{356}* could be mediated in *trans* by NS2B and 3_{181}, a truncated fragment of NS3 containing only the putative proteinase domain. Figure 3B illustrates results of cotransfection experiments with 3-5_{356}*

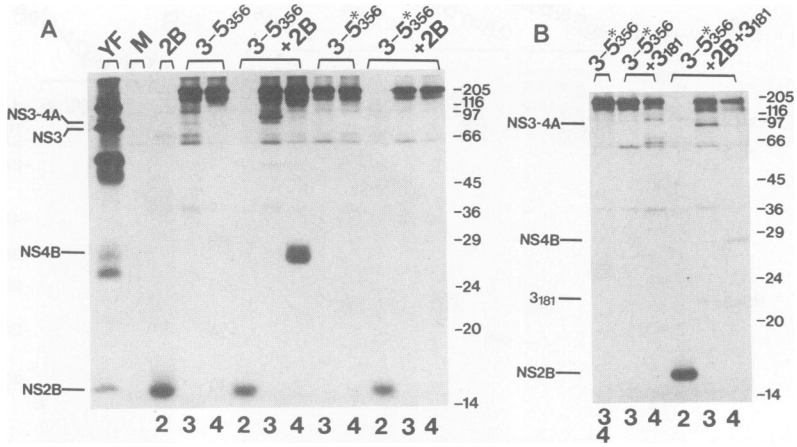

FIG. 3. NS2B is required for cleavage at the NS4B-5 site. Experimental procedures, YF and molecular weight markers, and the figure labels are as described in the legend to Fig. 2 and in Materials and Methods. (A) 3-5_{356} and 3-5_{356}* indicate transfection with 3-5_{356} or 3-5_{356}* alone; 2B + 3-5_{356} and 2B + 3-5_{356}* indicate cotransfection with NS2B and 3-5_{356} or NS2B and 3-5_{356}*. (B) 3_{181} + 3-5_{356}* indicates cotransfection with 3_{181} and 3-5_{356}*; 2B + 3_{181} + 3-5_{356}* indicates cotransfection with NS2B, 3_{181}, and 3-5_{356}*.

and either 3_{181} or a mixture of 3_{181} and NS2B as sources of proteinase. In the presence or absence of 3_{181}, the 3-5_{356}* polyprotein was expressed but no detectable cleavage products were generated. Cotransfection of 2B, 3_{181}, and 3-5_{356}* generated cleavage products including an 83-kDa NS3-specific protein and the 27-kDa NS4B protein. These results are consistent with the hypothesis that a specific interaction of NS2B with either the NS3 proteinase domain or the substrate or both is necessary for efficient cleavage at the 4B-5 site and that NS2B and NS3 can act in *trans*. In the absence of cleavage at the 4B-5 site, NS3-4A or a protein consistent in size with 4B-5_{356} was not observed in any of these experiments, suggesting that cleavage at the 4A-4B signalase site did not occur in the absence of cleavage at the 4B-5 site (see Discussion).

***trans* cleavage of other catalytically inactive substrates.** To examine *trans* cleavage at the other dibasic sites in the YF nonstructural region, we tested sig2A-5_{356}* as a substrate. Figure 4 illustrates the products generated after cotransfection of sig2A-5_{356}* with NS2B, 3_{181}, or 2B-3_{181}. Cotransfection of NS2B and sig2A-5_{356}* did not alter the pattern of proteins produced by sig2A-5_{356}* alone. Cotransfection of sig2A-5_{356}* and 3_{181} yielded the NS4B protein along with trace amounts of the 30- to 33-kDa NS2B-specific putative 2A-2B proteins resulting from cleavage at the NS2B-3 site (see also Fig. 2, sig2A-5_{356}). Additional cleavage products included a 150-kDa NS2B-, NS3-, and NS4B-specific polyprotein (presumably sig2A-4B or 2A-4B, predicted molecular masses of 154 and 150 kDa, respectively), and a 115-kDa NS2B- and NS3-specific protein (presumably 2A-4A, predicted molecular mass of 123 kDa). Although a minor 83-kDa NS3-specific species was present, a protein consistent in size with authentic NS3 was not observed. Cotransfection of sig2A-5_{356}* with 2B-3_{181} or with a mixture of NS2B and 3_{181} generated protein patterns similar to that described for 3_{181}. However, only with 2B-3_{181} was a protein migrating at the position of mature NS3 observed. It is unclear whether this apparent difference in cleavage efficiency at the 3-4A site reflects a quantitative difference in the level of active proteinase or a real difference in the substrate specificity of the proteinase generated by cotransfection of NS2B and 3_{181} versus the *cis*-cleaving 2B-3_{181} polyprotein. These results indicate that the NS3 proteinase domain can participate in *trans* cleavage at the 2B-3, 3-4A, and 4B-5 dibasic sites and that the efficiency of *trans* cleavage can differ at different sites.

***trans* cleavage at the NS2A-2B and NS2B-3 sites.** Results with sig2A-5_{356}* and 3_{181} cotransfection, in which NS2B was not detected, also suggested that the 2A-2B site was not cleaved efficiently in *trans*. To examine this more carefully, we performed cotransfection experiments using the 3_{181} proteinase and catalytically inactive $2A_{110}$-3_{181}* and 2B-3_{181}* substrates (Fig. 5A). For comparison, the cleavage products of 2B-3_{181} and $2A_{110}$-3_{181} are shown. Cotransfection of 3_{181} and 2B-3_{181}* produced minor amounts of NS2B,

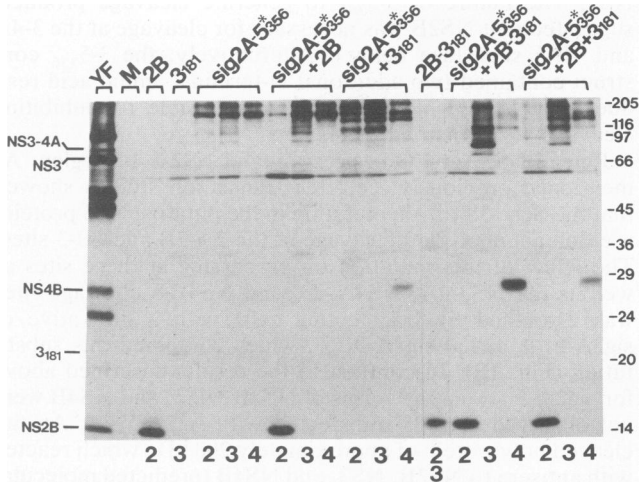

FIG. 4. Cotransfection experiments to examine *trans* cleavage of a catalytically inactive sig2A-5_{356}* substrate. Experimental procedures, YF and molecular weight markers, and the figure labels are as described in the legend to Fig. 2 and in Materials and Methods except that in this experiment, cells were labeled with [^{35}S]methionine for 5 h. 2B, 3_{181}, 2B-3_{181}, and sig2A-5_{356}* indicate transfection with these DNAs alone; sig2A-5_{356}* + 2B, sig2A-5_{356}* + 3_{181}, sig2A-5_{356}* + 2B-3_{181}, and sig2A-5_{356}* + 2B + 3_{181} indicate cotransfection experiments.

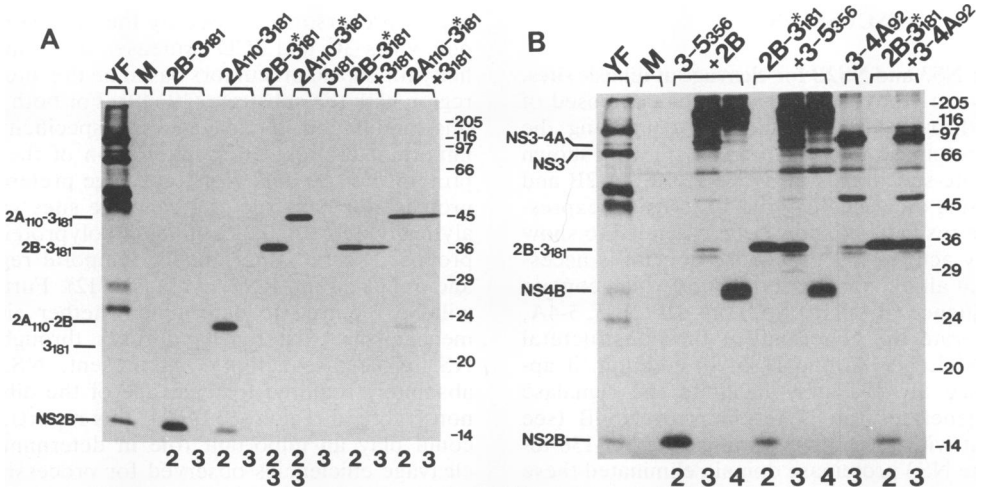

FIG. 5. *trans* cleavage reactions in the NS2-NS3 region. Experimental procedures, YF and molecular weight markers, and the figure labels are as described in the legend to Fig. 2 and in Materials and Methods. (A) 2B-3_{181}, $2A_{110}$-3_{181}, 2B-3_{181}*, $2A_{110}$-3_{181}*, and 3_{181} indicate transfection with the respective DNAs. 3_{181} + 2B-3_{181}*, and 3_{181} + $2A_{110}$-3_{181}* indicate cotransfection experiments. (B) 2B + 3-5_{356}, 2B-3_{181}* + 3-5_{356}, and 3-$4A_{92}$ + 2B-3_{181}* indicate cotransfection experiments. 3-$4A_{92}$ indicates transfection with this DNA alone.

indicating that 2B-3 cleavage had occurred. Transfection with 3_{181} and $2A_{110}$-3_{181}* produced a 23-kDa NS2B-specific protein consistent in size with $2A_{110}$-2B, but only trace amounts of NS2B or 2B-3_{181}* could be detected (which are not visible in the exposure shown in Fig. 5). Thus, for sig2A-5_{356}*, $2A_{110}$-3_{181}*, and 2B-3_{181}*, cleavage at the 2B-3 site can occur efficiently if 3_{181} is supplied in *trans*, but *trans* cleavage at the 2A-2B site was very inefficient. Similar results were also found in experiments with another catalytically inactive construct, sig2A-3_{78}, which contains a C-terminal deletion including NS3 Ser-138 (data not shown).

***trans* cleavage activity of NS3-containing polyproteins.** To determine whether the NS3 proteinase domain could function within the context of a polyprotein, we tested the 3-5_{356} and 3-$4A_{92}$ polyproteins for *trans* cleavage activity. Both constructs generated cleavage at the 2B-3 site with 2B-3_{181}* (Fig. 5B) and $2A_{110}$-3_{181}* substrates (data not shown). The NS2B-related cleavage products were identical to those described above with 3_{181}. Similar to the results found for 2B and 3-5_{356} cotransfection, NS4B was also observed with coexpression of 3-5_{356} and 2B-3_{181}*. This suggests either that 2B-3_{181}* can interact directly with 3-5_{356} to allow cleavage at the 4B-5 site or that NS2B produced from cleavage of 2B-3_{181}* by the proteinase domain supplied by 3-5_{356} participates in 4B-5 cleavage.

***trans* cleavage with nonoverlapping constructs.** Since the constructs used in the cotransfection experiments described above contained homologous DNA segments, we could not rigorously exclude the possibility that homologous recombination between plasmids was generating templates for production of polyproteins containing an active proteinase domain. Although this is extremely unlikely given the efficiencies observed in many of the cleavage reactions, we tested an additional catalytically inactive substrate, 3_{540}-5_{356}, in which only 84 C-terminal residues of NS3 remain. Figure 6 illustrates the results of transfection with 3_{540}-5_{356} alone or in combination with either 3_{181} or 2B-3_{181}. Transfection of 3_{540}-5_{356} or cotransfection with 3_{181} or 2B (data not shown) generated on NS4B-specific protein of 100 kDa (predicted molecular mass, 95 kDa) and no identifiable cleavage products. This protein (which comigrated with a mock protein) reacted with NS4B-specific antiserum but not NS3-specific antiserum, presumably because of lack of reactive NS3 epitopes in this deletion construct. In the presence of 2B-3_{181} (or 2B plus 3_{181}; data not shown), the NS4B protein was generated. These results provide additional evidence that both NS2B and an active proteinase domain are required for *trans* cleavage at the 4B-5 site and that the cleavages observed in these *trans* processing experiments are due to bimolecular reactions and not to recombination events generating a template encoding a polyprotein which is cleaved in *cis*.

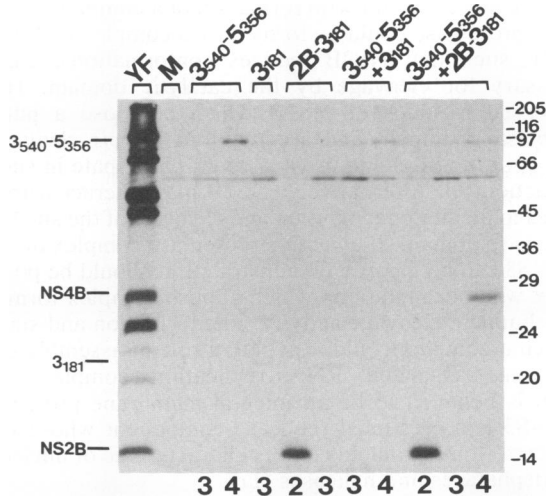

FIG. 6. *trans* cleavage reactions with a nonoverlapping proteinase and substrate. Experimental procedures, YF and molecular weight markers, and the figure labels are as described in the legend to Fig. 2 and in Materials and Methods. 3_{540}-5_{356}, 3_{181}, and 2B-3_{181} indicate transfection with the respective DNAs. 3_{540}-5_{356} + 3_{181} and 3_{540}-5_{356} + 2B-3_{181} indicate cotransfection experiments.

DISCUSSION

Requirement for NS3 and NS2B for cleavage at dibasic sites. In a previous in vitro study, YF polyproteins composed of the NS2 region through the NS3 domain containing the proposed serine proteinase catalytic triad (1, 16) were shown to be capable of site-specific *cis* cleavage at the 2A-2B and 2B-3 sites (11). Using the vaccinia virus-T7 transient expression system, these results have now been extended to show that a catalytically active NS3 proteinase domain is necessary for cleavage at all the nonstructural sites which contain the consensus sequence G(A)RR ↓ S(G) (2A-2B, 2B-3, 3-4A, and 4B-5) to generate the N termini of the nonstructural proteins NS2B, NS3, NS4A, and NS5. In addition, it appears that cleavage at 4B-5 may facilitate the signalase cleavage which generates the N terminus of NS4B (see below). Consistent with these observations, the Ser-138-to-Ala mutation in the NS3 proteinase domain eliminated these cleavages. The data also demonstrated that the proposed NS3 proteinase domain can function efficiently in *trans* for cleavage at two sites (2B-3 and 4B-5) and that NS2B, either derived from a catalytically active polyprotein or supplied in *trans*, is also required for cleavage at the 3-4A and 4B-5 sites. Together with results recently reported for DEN4, in which both NS2B and the putative proteinase domain were required for cleavage at the 3-4A site and could function in *trans* for cleavage at the 2A-2B and 2B-3 sites (14), these data suggest that both NS2B and the proteinase domain are required for cleavage at all the dibasic sites in the flavivirus nonstructural polyprotein. This requirement for NS2B is consistent with previous observations in cell-free and transient expression assays in which it was shown that in-frame deletions in NS2B abolish site-specific cleavage at the 2A-2B and 2B-3 cleavage sites of DEN2 (31), DEN4 (14), and YF (6).

At present, the interactions of the NS3 proteinase domain, NS2B, and the cleavage substrates have not been defined. The transient expression data for YF (this report) and DEN4 (14), as well as the results of in vitro studies which demonstrated that cleavage at the 2A-2B site of YF was dilution insensitive even though cleavage at the 2B-3 site occurred first, are all consistent with formation of a complex (11). The active proteinase could be formed by a complex of NS3 and NS2B, such that NS2B induces conformational changes necessary for cleavage by the catalytic domain. Highly conserved residues of NS2B which compose a putative cytoplasmic domain and are required for *cis* cleavage of $2B\text{-}3_{181}$ at the 2B-3 site in vitro could participate in such an interaction (6). Alternatively, NS2B may interact with substrates to facilitate recognition and cleavage of the site by the proteinase domain. If an active proteinase complex of NS2B and NS3 can be directly demonstrated, it should be possible to ask whether mutations which abolish complex formation also eliminate cleavage activity. The formation and stability of such a complex could also play a role in assembly of the membrane-associated RNA replication complex, since NS2B is believed to be an integral membrane protein (46) and NS3 is a presumed replicase component whose C-terminal two-thirds contains motifs characteristic of nucleoside triphosphatases and helicases (17).

In other viral systems, examples exist in which dimerization is required for proteinase activity (for example, see reference 20) or in which interaction of the catalytic domain of the proteinase with additional viral polypeptides can affect cleavage site specificity. In cowpea mosaic virus, the specificity of the 24-kDa protease for *trans* cleavages of the capsid precursor is altered by the 32-kDa protein (44). The poliovirus 3C and 3CD proteases differ in cleavage specificity for capsid precursors based on the interaction of the 3D region with the substrate (19, 49). For both of these viruses, this modulation of cleavage site specificity is presumably important for regulating maturation of the structural polyprotein. For Sindbis virus, cleavage preference of the nsP2 protease for nonstructural cleavage sites varies among catalytically active nsP2-containing polyproteins and has been proposed to be important for temporal regulation of plus- and minus-strand RNA synthesis (12). Further experiments will be required to determine whether similar regulatory mechanisms exist for flaviviruses through modulation of NS3 proteinase activity. At present, NS2B seems to be absolutely required for cleavage of the dibasic sites in the nonstructural region (6, 14, 31; this report), but this protein could play an important role in determining the different cleavage efficiencies observed for processing at these sites (see below).

Polyprotein processing in transient assays compared with YF-infected cells. In contrast to processing studies in cell extracts (11, 31, 45), dilution analysis and useful kinetic studies are not possible with this vaccinia virus-T7 expression system. Thus, the actual cleavage mechanisms occurring in these transient expression assays or in virus-infected cells cannot be rigorously determined at this time. With these limitations in mind, several observations concerning polyprotein processing in the transient assays reported here are worth noting.

All dibasic cleavage sites, as well as the proposed 4A-4B signalase site, were cleaved with the catalytically active polyproteins $sig2A\text{-}5_{356}$, $2A_{110}\text{-}5_{356}$, and $2B\text{-}5_{356}$. In addition, the $3_{540}\text{-}5_{356}$ substrate which was cleaved efficiently in *trans* at the 4B-5 site was also cleaved at the 4A-4B site, presumably by signalase. This suggests that the structural differences between these polyproteins do not dramatically affect cleavage at these sites and, in particular, that an N-terminal signal sequence is not required for these cleavages. This result is somewhat surprising since NS2A, NS2B, NS4B, and probably NS4A are believed to be integral membrane proteins (5, 46). We have not determined whether these cleavages occur in association with membranes in the vaccinia virus-T7 system, but it seems likely that proper membrane topology for the NS2 and NS4 regions can be generated independent of an N-terminal signal sequence. Alternatively, at least for the NS2 region, proper membrane association may not be necessary since previous studies demonstrated that cleavages at the 2A-2B and 2B-3 sites can occur in vitro in the absence of microsomal membranes (11).

It was noticed, for $sig2A\text{-}5_{356}$ and $2A_{110}\text{-}5_{356}$, that cleavage at the 2B-3 site was more efficient than cleavage at the 2A-2B site. It is possible that interaction between NS1 and NS2A, which are present as an identifiable NS1-2A precursor in YF-infected cells (9), is necessary for efficient cleavage at the 2A-2B site. In addition, alternative processing appeared to have occurred in the NS2A region, resulting in 2A-2B-related products which were smaller than predicted. Some of these products have occasionally been observed in YF-infected cells (6) and may result from alternative processing in the NS2A region prior to cleavage at the 2A-2B site (8, 9). In any case, our hypothesis, based on the previous in vitro data, the failure to observe 2B-3-containing polyproteins with self-processing polyproteins (Fig. 2A), and incomplete *trans* cleavage of 2B-3-containing substrates (Fig. 5A), is that *cis* cleavage predominates at the 2A-2B and the 2B-3 sites. However, the studies reported here, as well as recent

data from in vitro assays (6), show that *trans* cleavage can also occur at these sites but with very inefficient cleavage at the 2A-2B site relative to the 2B-3 site.

Processing of the NS3-4-5 region also appears to be complex, and similarities as well as significant differences were observed in these transient expression experiments compared with processing in infected cells. In YF-infected cells, the NS3-4A cleavage site (GRR↓G) was cleaved rather efficiently, based on the detection of NS3 as well as an unstable 39-kDa NS4A-4B polyprotein whose N terminus has been defined by sequence analysis (9). However, two additional NS3-specific species which do not react with NS2B antiserum were also observed: a short-lived 83-kDa NS3-specific protein, corresponding in size to intact NS3-4A, and a stable ~75-kDa protein which is believed to result from an alternative cleavage in the 4A region. These results suggest that alternative cleavage pathways exist for processing of the NS3-4-5 region (9). In the transient expression experiments with either catalytically active polyproteins or cotransfection assays for *trans* cleavage, cleavage at the 3-4A site, as assayed by the ratio of NS3-4A to NS3, appeared to be much less inefficient than that in YF-infected cells. Although this ratio could be misleading if mature NS3 was degraded rapidly in vaccinia virus-infected cells, this explanation seems unlikely since NS3 can be expressed in a stable form by using the vaccinia virus system (10). Although we cannot exclude the possibility that vaccinia virus infection affects cleavage efficiency at the 3-4A site, it seems more likely that additional sequences in the YF polyprotein, which were absent in these constructs, may influence cleavage efficiency at this site. In any case, the experiments indicating that *trans* cleavage of the 3-4A site occurs only inefficiently may in part explain the presence of NS3-4A in YF-infected cells. Putative NS3-4A polyproteins, which have been observed for several different flaviviruses, could be functionally distinct from NS3 since they would contain C-terminal hydrophobic sequences derived from NS4A, which represent potential membrane-spanning domains. Our hypothesis for an alternative pathway leading to generation of minor but stable NS3-4A products involves infrequent cleavage at the 4A-4B signalase site prior to cleavage at the 3-4A site, resulting in a polyprotein conformation which does not favor *cis* or *trans* cleavage at the 3-4A site. Inefficient cleavage at the YF 3-4A site relative to other dibasic sites could also involve the charged polar (Glu) residue at the P4 position of the consensus cleavage site instead of the nonpolar (Ala, Phe) or uncharged polar (Ser, Thr, Gly) residues usually found at this position (see reference 7 for a review).

In contrast to the 3-4A site, the 4B-5 site was cleaved efficiently in catalytically active polyproteins and in *trans*. This is consistent with the rapid disappearance of NS4B-specific polyproteins in YF-infected cells (9). An interesting observation was that at least for polyproteins extending through the first 356 residues of NS5, cleavage at the 4B-5 site appears to be a necessary prerequisite for signalase cleavage at the 4A-4B site. We have observed, however, that a construct containing only 88 N-terminal amino acid residues of NS5 (sig2A-5_{88}) undergoes inefficient cleavage at the 4B-5 site but generates a protein corresponding to 4B-5_{88}, consistent with cleavage at the 4A-4B signalase site (25). This suggests that in the absence of 4B-5 cleavage, as in the context of the NS3 Ser-138-to-Ala mutation or with polyproteins lacking NS2B, NS5 sequences beyond amino acid residue 88 may inhibit the ability of the NS4A-4B region from assuming a conformation which allows cleavage by signalase. Hence, efficient 4A-4B cleavage can be observed either by allowing prior cleavage at the 4B-5 site or by deleting NS5 sequences. The data with sig2A-5_{88} also indicate that NS5 sequences can influence cleavage at the 4B-5 site. It is unknown whether this result is due to inhibition of cleavage at the 4B-5 site mediated by the shortened NS5 polypeptide or whether it reflects a requirement for additional sequences in NS5 (between residues 88 and 356) for efficient cleavage at the 4B-5 site.

Clearly, additional studies are needed to define the determinants of cleavage site specificity, the protein-protein interactions involved in determining cleavage site preferences and order of cleavage, and the relevance of these *cis* and *trans* cleavage events for assembly of functional viral RNA replication complexes. However, the results presented here have delineated two discrete domains of the YF polyprotein, NS2B and 3_{181}, which appear to be the only viral sequences necessary for *trans* cleavage at the dibasic sites in the nonstructural polyprotein. Studies can now be directed toward development of *trans* assays for site-specific cleavage in vitro and their use for purification of these domains for further structural and biochemical characterization.

ACKNOWLEDGMENTS

We thank our colleagues, especially Peter J. Bredenbeek and Ann Nestorowicz, for helpful suggestions on the manuscript, Joel Dalrymple for providing mouse hyperimmune antiserum to YF, and Peter Mason for providing BHK-21-15 cells and the protocol for DNA transfection.

This work was supported by grants from the Pew Memorial Trust and the Public Health Service (AI31501) and U.S. Army contract DAMD17-87-C-7154. C.M.R. is a Pew Scholar in the Biomedical Sciences. T.J.C. was supported in part by Public Health Service grants AI07739 and AI00973.

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