Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8898–8902, November 1990
Biochemistry

Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein

(flavivirus/protein processing/trypsin superfamily/site-directed mutagenesis/catalytic triad)

THOMAS J. CHAMBERS[†], RONALD C. WEIR[†‡], ARASH GRAKOUI[†], DAVID W. MCCOURT[§], J. FERNANDO BAZAN[¶], ROBERT J. FLETTERICK[¶], AND CHARLES M. RICE[†‖]

[†]Department of Molecular Microbiology, Box 8230, and [§]Howard Hughes Medical Institute, Washington University School of Medicine, 660 South Euclid Avenue, Saint Louis, MO 63110-1093; and [¶]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448

Communicated by Carl Frieden, August 23, 1990 (received for review May 25, 1990)

ABSTRACT **Sequence homology and molecular modeling studies have suggested that the N-terminal one-third of the flavivirus nonstructural protein NS3 functions as a trypsin-like serine protease. To examine the putative proteolytic activity of NS3, segments of the yellow fever virus genome were subcloned into plasmid transcription/translation vectors and cell-free translation products were characterized. The results suggest that a protease activity encoded within NS2B and the N-terminal one-third of yellow fever virus NS3 is capable of cis-acting site-specific proteolysis at the NS2B-NS3 cleavage site and dilution-insensitive cleavage of the NS2A-NS2B site. Site-directed mutagenesis of the His-53, Asp-77, and Ser-138 residues of NS3 that compose the proposed catalytic triad implicates this domain as a serine protease. Infectious virus was not recovered from mammalian cells transfected with RNAs transcribed from full-length yellow fever virus cDNA templates containing mutations at Ser-138 (which abolish or dramatically reduce protease activity *in vitro*), suggesting that the protease is required for viral replication.**

Yellow fever virus (YF), the prototype member of the family *Flaviviridae*, contains a single molecule of positive-stranded RNA ≈11 kilobases long (1). The gene order is 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ where C, prM, and E denote the structural protein precursors and NS1 through NS5 represent the nonstructural proteins (NSs). A single long open reading frame encodes these proteins, which are produced by proteolytic cleavage (1, 2). It has been proposed that the structural protein precursors and the N terminus of NS4B are processed cotranslationally by host signalase in association with membranes of the endoplasmic reticulum (3, 4). In contrast, cleavages generating the N termini of the nonstructural proteins NS2B, NS3, NS4A, and NS5, which follow dibasic amino acid residues (for review, see ref. 5), occur rapidly and efficiently in YF-infected cells (3) and are proposed to be mediated by a viral protease located in the cytoplasm (ref. 1; for review, see ref. 5).

A number of positive-stranded RNA viruses encode protease domains (for review, see ref. 6) that are homologous to the cellular trypsin-like serine proteases (see ref. 7). Among the flaviviruses, sequence homology and molecular modeling studies have predicted that the N-terminal 180 amino acids of the large, highly conserved NS3 protein contain a serine protease-like domain (8, 9). The positions of three amino acid residues of YF NS3 (His-53, Asp-77, and Ser-138) are strictly conserved among flaviviruses and correspond spatially to the catalytic triad of the trypsin-like serine proteases.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

In this report we have obtained evidence for a protease activity encoded by the YF NS2B-NS3 region, mutagenized the histidine, aspartic acid, and serine residues in the proposed NS3 catalytic triad, and have studied the effects of mutations that abolish or diminish the *in vitro* cleavage activity on YF infectivity.

MATERIALS AND METHODS

Cell Culture and Virus Infection. Growth of BHK-21 cell monolayers and their infection with the YF 17D strain were carried out as described (10).

Construction of Transcription Vectors. DNA cloning was done using standard procedures (11). The transcription vector pET8C (12) contains a promoter for T7 RNA polymerase, followed by a unique *Nco* I site (CCATGG) with the ATG in an appropriate context for either prokaryotic or eukaryotic expression (12). Regions of YF cDNA were subcloned into pET8C using this *Nco* I site and a *Bam*HI site preceding the T7 terminator (12). For construction of pET8C-NS2B3.1, YFM5.2 DNA (13) was subjected to polymerase chain reaction amplification using two synthetic oligonucleotide primers (14) that positioned an *Nco* I site upstream from the NS2B N terminus and a termination codon (UAA), followed by a *Bgl* II restriction site after amino acid 181 of NS3. Amplified fragments were partially digested with *Nco* I (NS2B contains an internal *Nco* I site) and *Bgl* II and inserted into the pET8C vector after digestion with *Nco* I and *Bam*HI. The structure of the polymerase chain reaction-amplified region was verified by sequencing. For construction of the pET8C-NS2A*2B3.1 plasmid, the 284-base-pair *Nco* I fragment of pET8C-NS2B3.1 was replaced with the 623-base-pair *Nco* I fragment (YF nucleotides 3510–3835) from pYFM3.3 (13).

Full-length YF cDNA templates for *in vitro* transcription were constructed by *in vitro* ligation of restriction fragments from pYFM5.2 or mutant derivatives and pYF5′3′IV plasmids (13). The pYFM5.2 derivatives with mutations in the NS3 protease domain were constructed by replacing the *Sst* I fragment of pYFM5.2 (YF nucleotides 4335–5111) with the corresponding *Sst* I fragment from the clones produced by site-directed mutagenesis (see below).

Site-Directed Mutagenesis. Mutations in the putative protease domain were made by oligonucleotide-directed mutagenesis using uridylated (15) phagemid DNA. The phagemid was a derivative of pET8C-NS2B3.1, called pET/BS(+) NS2B3.1, constructed by subcloning the *Fsp* I–*Sca* I fragment of pBluescript II SK(+) (Stratagene) containing the f1

Abbreviations: YF, yellow fever virus; NS, nonstructural protein.

[‡]Present address: Department of Biochemistry, Faculty of Science, The Australian National University, Canberra, A.C.T. 2601, Australia.
[‖]To whom reprint requests should be addressed.

origin into pET8C-NS2B3.1 that had been digested with *Eco*RV and *Sca* I. The strand rescued with M13 helper phage corresponded to the YF plus strand. Mutations, verified by sequence analysis, were as follows: His-53 → Ala (CAU → GCU), Asp-77 → Asn (GAC → AAC), Asp-77 → Ala (GAC → GCU), Ser-138 → Ala (UCA → GCU), and Ser-138 → Cys (UCA → UGC).

***In Vitro* Transcription and Transfection.** The 5′ capped RNA transcripts were synthesized using T7 or SP6 RNA polymerase and linearized DNA templates (13). Transfection of BHK-21 cells and plaque assay for YF were performed as described (13).

Cell-Free Translation and Protein Analyses. Cell-free translation was performed using rabbit reticulocyte lysate (Promega) and following the manufacturer's specifications. Proteins were labeled by inclusion of [^{35}S]methionine, [^{3}H]leucine, [^{3}H]valine, or [^{3}H]tryptophan (Amersham). Translation products were immunoprecipitated with NS2B- and NS3-specific rabbit antisera (3, 10). SDS/PAGE, fluorography, and partial N-terminal amino acid sequencing were performed essentially as described (3).

RESULTS

Cell-Free Expression of a Protease Activity Cleaving at NS2A-NS2B and NS2B-NS3. Fig. 1 illustrates the region of the YF polyprotein represented in the pET8C transcription vectors pET8C-NS2B3.1 and pET8C-NS2A*2B3.1 The primary translation product derived from transcripts of pET8C-NS2B3.1 (denoted NS2B3.1) includes two extra N-terminal amino acids (Met-Ala), the entire NS2B protein, and the first 181 amino acids of NS3 that contains the putative protease domain. The primary translation product from pET8C-NS2A*2B3.1 (denoted NS2A*2B3.1) contains an additional N-terminal extension into the NS2A protein that includes the last 115 amino acids of NS2A and the proper NS2A-NS2B cleavage site. Possible protease activity associated with these regions of the YF polyprotein was examined by translating capped RNA transcripts in a rabbit reticulocyte lysate.

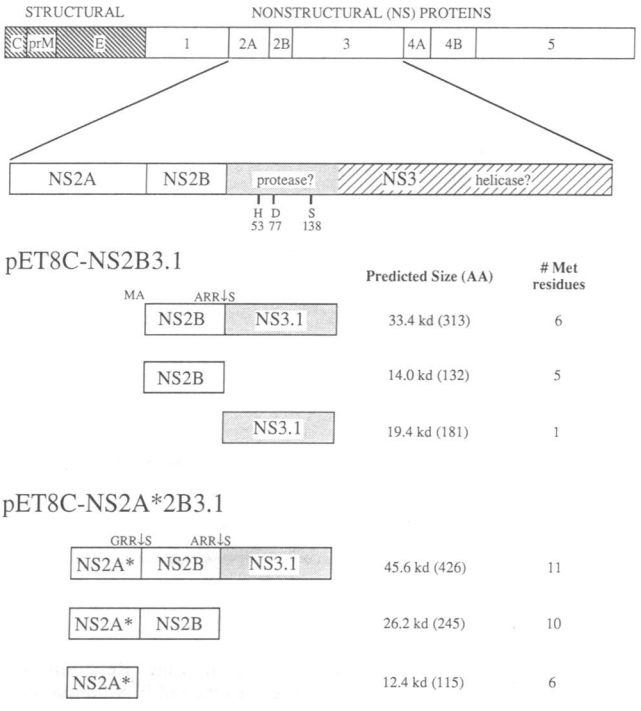

FIG. 1. Regions of the YF polyprotein utilized for *in vitro* expression. The top line indicates the YF genome organization. Below is the region encoding the proteins NS2A, NS2B, and NS3. Shaded and striped portions of NS3 represent the putative protease and helicase domains, respectively, and the residues of the proposed catalytic triad of the protease are indicated (8, 9). The pET8C constructs are described in the text. AA, amino acid(s); kd, kDa.

Fig. 2 illustrates the translation products of pET8C-NS2B3.1 and pET8C-NS2A*2B3.1 RNAs immunoprecipitated with antisera specific to the NS2B and NS3 proteins. For pET8C-NS2B3.1, the sizes and immunoreactivity of the translation products were consistent with their identification as NS2B3.1 (35 kDa), NS3.1 (21 kDa), and NS2B (15 kDa). A 33-kDa protein that reacted with both antisera was not characterized further. These products were not observed in translations performed in the absence of exogenous RNA. Translation in the presence of canine microsomal membranes did not significantly change the protein pattern (data not shown). The N-terminal sequence of the 21-kDa protein (Fig. 3) corresponded to the authentic N terminus of NS3 generated *in vivo* (2).

Translation of pET8C-NS2A*2B3.1 RNA yielded products whose sizes and immunoreactivity were consistent with their identification as NS2A*2B3.1 (43 kDa), NS2A*2B (23 kDa), NS3.1 (21 kDa), and NS2B (15 kDa) (Fig. 2). The NS2A* protein was not identified due to lack of an appropriate antiserum. The addition of microsomal membranes increased the amount of the 23-kDa protein but did not otherwise change the protein pattern (data not shown). The N-terminal sequence of the 15-kDa protein (Fig. 3) corresponded to the N terminus of authentic YF NS2B (10). These results are consistent with the hypothesis that a proteolytic activity residing within NS2B-NS3.1 mediates site-specific cleavage at the authentic NS2A-NS2B and NS2B-NS3 cleavage sites.

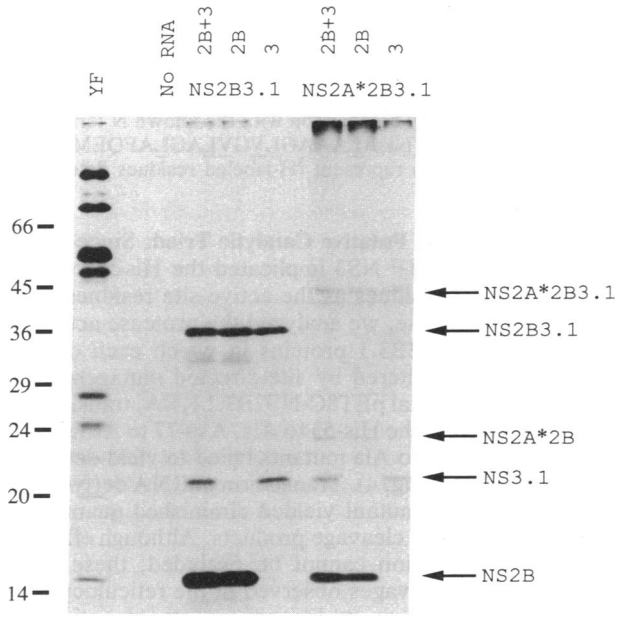

FIG. 2. Identification of *in vitro* translation products of transcripts from pET8C-NS2B3.1 and pET8C-NS2A*2B3.1. Reaction mixtures contained ≈100 ng of RNA in the presence of [^{35}S]-methionine at 30°C. Reactions were terminated after 30 min (NS2B3.1) or 60 min (NS2A*2B3.1) by addition of SDS to 0.5% and proteins were immunoprecipitated and analyzed by SDS/PAGE using 14% gels, followed by fluorography. Lanes: YF, [^{35}S]methionine-labeled YF-specific protein markers from infected BHK-21 cells (3); No RNA, reaction performed without exogenous RNA immunoprecipitated with a mixture of antiserum specific for NS2B and NS3; 2B+3, samples immunoprecipitated with a mixture of antiserum to NS2B and NS3; 2B and 3, samples immunoprecipitated with antiserum to NS2B or NS3, respectively. Positions of molecular mass markers (determined by staining with Coomassie blue) are indicated at left in kDa.

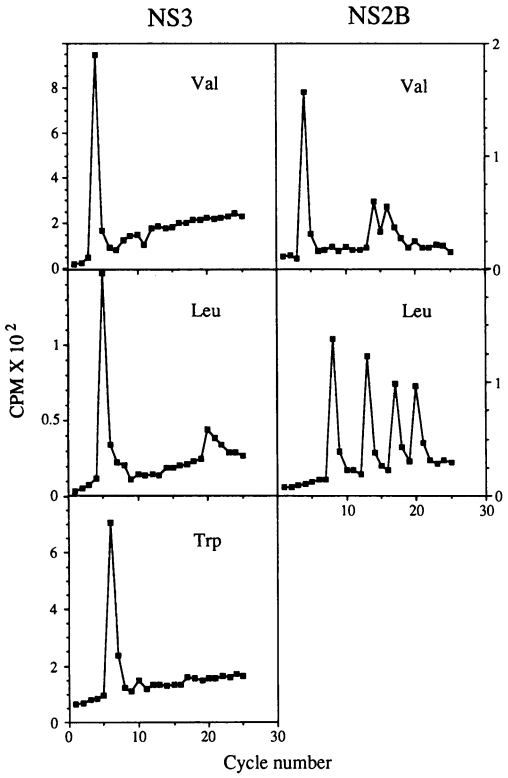

FIG. 3. N-terminal amino acid sequence analysis of YF NS3.1 and NS2B produced by cleavage *in vitro* during cell-free translation of RNA transcripts derived from pET8C-NS2B3.1 and pET8C-NS2A*2B3.1. The graphs show cpm of ^3H recovered after each cycle of Edman degradation. The N terminus of NS3.1 is, by comparison with the known N terminus of YF NS3 (1, 16), SGDVLW DIPTPKIIEECEHLEDGIY, where underlined amino acids represent ^3H-labeled residues detected by sequence analysis. The N terminus of NS2B is, by comparison with the known N terminus of YF NS2B (1, 10), SIPVNEALAAAGLVGVLAGLAFQEM, where underlined amino acids represent ^3H-labeled residues detected by sequence analysis.

Mutagenesis of the Putative Catalytic Triad. Since homology of trypsin and YF NS3 implicated the His-53, Asp-77, and Ser-138 NS3 residues as the active site residues of the putative NS3 protease, we analyzed the protease activity *in vitro* of mutant NS2B3.1 proteins in which each of these residues had been altered by site-directed mutagenesis. In contrast to the parental pET8C-NS2B3.1 RNA, translation of RNAs derived from the His-53 to Ala, Asp-77 to Asn, Asp-77 to Ala, and Ser-138 to Ala mutants failed to yield detectable cleavage products (Fig. 4). Translation of RNA derived from the Ser-138 to Cys mutant yielded diminished quantities of the NS3.1 and NS2B cleavage products. Although effects on substrate conformation cannot be excluded, these results suggest that the cleavages observed in the reticulocyte system are not due to an endogenous protease activity. In addition, the effect of substitutions at His-53, Asp-77, or Ser-138 on the cleavage efficiency *in vitro* suggests that these residues are important for catalytic activity although a role for NS2B in modulating catalytic function is not excluded.

Effect of Dilution on NS2B3.1 Cleavage Activity. If the cleavages observed *in vitro* occur in cis, then dilution of the translation products should have little effect on cleavage efficiency (17). Fig. 5 illustrates the effect of dilution of the cleavage activity of the protease encoded by NS2B3.1. In a pulse–chase protocol, as NS2B3.1 and the 33-kDa protein disappeared, the levels of the NS2B and NS3.1 increased, suggesting a precursor–product relationship. This processing reaction was insensitive to dilution over a 40-fold range. These results suggest cis-acting proteolysis at the NS2B-NS3

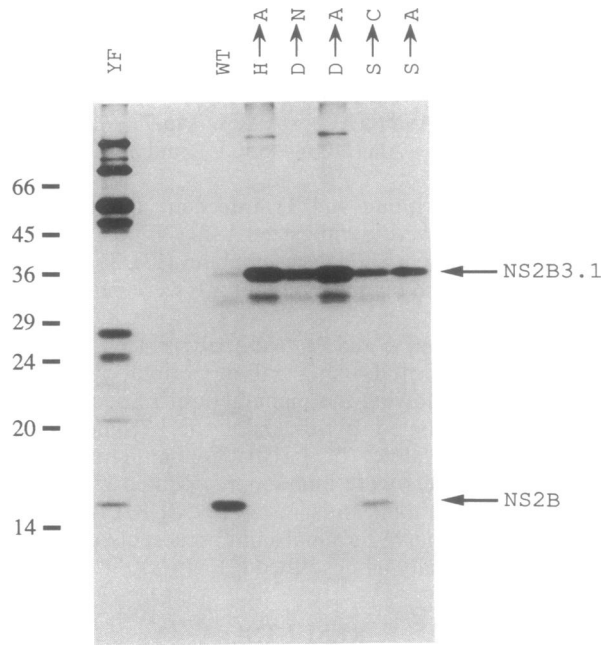

FIG. 4. Cleavage of NS2B3.1 products containing altered residues in the proposed catalytic triad. Reaction mixtures contained 100–200 ng of RNA in the presence of [^{35}S]methionine at 30°C. After 30 min, proteins were immunoprecipitated with a mixture of antiserum to NS2B and to NS3, and samples were analyzed by SDS/PAGE on 14% gels, followed by fluorography. Lanes: WT, parental YF sequence; H → A, D → A, D → N, S → C, S → A, mutations described in the text, but using the single letter code for amino acids; YF, YF protein markers. Positions of molecular mass markers are indicated at the left in kDa.

cleavage site but do not rule out an efficient trans cleavage. The latter alternative is not favored since trans cleavage of catalytically inactive substrates containing the NS2B-NS3

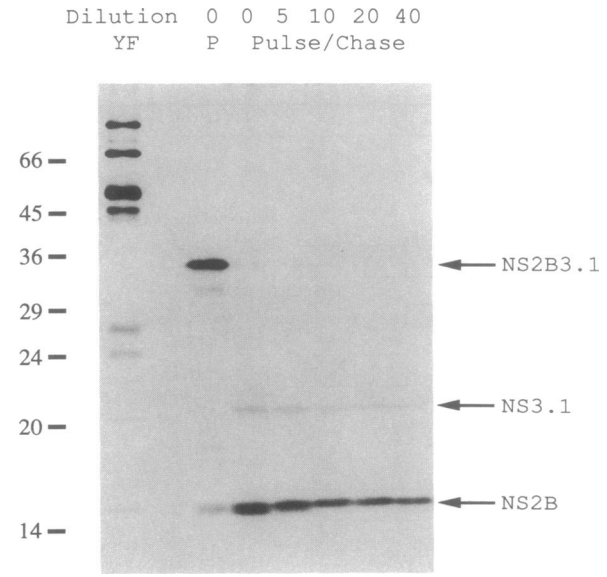

FIG. 5. Effect of dilution on NS2B3.1 cleavage. Reaction mixtures contained ≈100 ng of RNA in the presence of [^{35}S]methionine. After incubation at 30°C for 15 min, cycloheximide and unlabeled methionine were added to 100 μg/ml, and incubation was continued for 30 min. Diluted samples (0, 1:5, 1:10, 1:20, 1:40) were prepared by adding a portion of the reaction mixture to blank reticulocyte lysate that had been treated in the same manner. Proteins were analyzed as described in Fig. 2. Lane YF indicates YF protein markers. Positions of molecular mass markers are indicated at the left in kDa.

Table 1. Effect of mutations at Ser-138 of NS3 on infectivity of YF RNA transcripts

Mutation	pfu
Ser	60
Ser → Ala	0
Ser → Cys	0

RNA transcripts were derived from full-length cDNA templates containing serine, alanine, or cysteine at NS3 position 138. Relative quantities of full-length RNA as determined by agarose gel electrophoresis appeared identical for the three preparations. Plaque-forming units (pfu) on BHK-21 monolayers, produced per 30 ng of total RNA transcribed from *in vitro*-ligated full-length YF cDNA templates are shown, extrapolated from values obtained from infectious center assays performed in the range of 10 ng.

cleavage site has not been demonstrable *in vitro* (data not shown).

A similar experiment using the NS2A*2B3.1 protein demonstrated that production of NS2B from this polyprotein was also insensitive to dilution (data not shown). In addition, for NS2A*2B3.1, an NS2B3.1 species (35 kDa) was not observed (Fig. 2), suggesting that the NS2B-NS3 cleavage can occur prior to the NS2A-NS2B cleavage.

Effect of Ser-138 Mutations on YF Infectivity. Two mutations that either abolish (Ser-138 to Ala) or significantly reduce (Ser-138 to Cys) the NS2B3.1 cleavage activity *in vitro* were analyzed *in vivo* by transfection of BHK cells with RNA transcripts derived from full-length YF cDNA templates containing these mutations. In contrast to RNA transcripts derived from parental YF clones, no plaques were observed after transfection with these mutant RNAs (Table 1). If these mutations were deleterious for virus replication then leaky revertants would be expected to arise at some frequency. Revertants capable of forming plaques were not found, suggesting an early block, perhaps in viral RNA replication. However, direct measurements of virus-specific RNA and protein synthesis in cells transfected with these mutant RNAs will be required to elucidate the primary defect(s).

DISCUSSION

This study provides strong evidence that a protease activity is encoded within the YF NS2B-NS3 region, with specificity for the Gly-(Ala)-Arg-Arg↓Ser cleavage sites producing the N termini of NS2B and NS3. *In vitro*, the cleavage activity is rapid, relatively efficient, and, at least for this subregion of the YF polyprotein, not dependent on the addition of microsomal membranes. The data indicate that the NS2B-NS3 site is cleaved in cis followed by a dilution-insensitive cleavage at the NS2A-NS2B site. Results of site-directed mutagenesis of the His-53, Asp-77, and Ser-138 NS3 residues are consistent with the hypothesis that the catalytic activity resides in the NS3 domain and that these residues comprise a serine protease-like catalytic triad (see below). Formal proof that NS3 functions as a serine protease awaits purification of the active enzyme and determination of a high-resolution structure for the protease domain. Our results suggest that this protease may play an essential role in virus replication since a catalytically active domain appears to be necessary for the recovery of infectious virus. However, the participation of this NS3 domain in other essential viral functions that were disrupted by the mutations at Ser-138 cannot be excluded.

Evidence That NS3 Is a Trypsin-Like Serine Protease. This work tests the trypsin-like framework as a viable model for the structure and catalytic activity of the YF NS3 protease domain that mediates site-specific cis-acting cleavage at the NS2A-NS2B and NS2B-NS3 sites *in vitro*. The current understanding of the structure and function of serine proteases implicates a catalytic triad of histidine, aspartic acid, and serine residues in the hydrolysis of substrate peptide bonds (for review, see ref. 18). Comparative crystallographic analysis has revealed that the geometry of this catalytic triad remains invariant in the frameworks of evolutionarily unrelated serine proteases and lipases (18–21). Individual catalytic contributions of the triad residues have been probed by mutagenic replacement in cellular enzymes: for example, the substitution of histidine and serine with alanine in *Bacillus amyloliquifaciens* subtilisin (22) or of serine with cysteine rat anionic trypsin (23) results in significant decreases in enzymatic activity. For YF NS3, mutagenesis of NS3 His-53 and Ser-138 to Ala and of Asp-77 to Asn or Ala independently abolishes detectable proteolytic activity in this *in vitro* assay; in contrast, the Ser-138 to Cys mutation measurably decreases protease activity but is not lethal for the enzyme. These results are consistent with the proposed essential role in catalysis of these residues. Similar substitutions have been studied in the proposed serine protease catalytic triad (9) of the Sindbis virus capsid protein autoprotease (24). This cleavage, like the YF NS2B-NS3 cleavage, is rapid and occurs in cis; and substitutions of cysteine or threonine but not alanine or isoleucine for the proposed nucleophilic serine still permits cleavage (24). Mutagenesis experiments have also been carried out with viral cysteine proteases related to the YF NS3 enzyme: Dougherty *et al.* (25), Ivanoff *et al.* (26), and Cheah *et al.* (27) have mutated residues of the putative catalytic triad (7) of the tobacco etch virus, poliovirus, and human rhinovirus cysteine proteases, respectively. In particular, the substitution of the nucleophilic cysteine by serine in the tobacco etch virus 49-kDa protease, a converse experiment to the serine to cysteine mutations in YF NS3 (this work) and rat trypsin (23), results in an enzyme with decreased activity.

NS3 Cleavage Site Preferences. Cellular enzymes closely related to pancreatic trypsin retain a unique specificity for basic residues in the substrate binding pocket based on the electrostatic interaction of a conserved aspartic acid residue with the bound arginine/lysine substrate residue (28). Flavivirus NS3 proteases are predicted to conserve a spatially equivalent Asp-132 (YF numbering) residue, which may in part explain the specificity of these proteases for cleavage at sites after two basic amino acid residues (8). Other residues in the NS3 protease and its substrates must also be important in determining the restricted specificity of this protease as compared to pancreatic trypsin. Our results show that this specificity is unchanged for polypeptides containing only NS2B and the first 181 residues of NS3. Mutagenesis of the protease domain (by analogy to trypsin; refs. 18 and 29) as well as the cleavage sites can now be used to define specific residues important for this specificity. Since the NS3 protease domains and the nonstructural protein cleavage sites are highly conserved among flaviviruses (for review, see ref. 5) and the genetic data suggest that the YF NS3 protease is essential for virus production (this study), inhibitors specific for the NS3 protease, if they can be identified or designed, may be useful for general and effective antiviral therapy in flavivirus infections.

Implications for Processing of the YF Polyprotein. The current model for flavivirus polyprotein processing implicates the NS3 protease domain in cleavage of the sites that generate the N termini of NS2B, NS3, NS4A, and NS5 (for review, see ref. 5). The NS3 protease may also mediate cleavage of the anchored capsid protein to produce the mature form associated with virus particles (5, 30), thus playing an important role not only in production of the RNA replicase components but also in virion assembly.

If the data presented herein for cleavage *in vitro* can be extrapolated to *in vivo* processing, then the nascent NS3

protease domain should efficiently mediate the cytoplasmic cleavages necessary to generate NS2B essentially in cis. These results are consistent with the inability to detect NS2B-related precursors in pulse–chase analyses of YF-infected cells (3). The *in vitro* data also suggest that NS2A*2B and NS3.1 remain associated as a complex such that the slower NS2A*-NS2B cleavage is preferentially catalyzed by the NS3.1 protease domain present in the original polyprotein. This raises the intriguing possibility that these polypeptides might be important in modulating the activity of the NS3 protease domain. This is supported by recent experiments that show that both NS2B and NS3 are required for cleavage at the NS4B-NS5 site (T.J.C. and C.M.R., unpublished results).

At this point it is unclear whether the preference observed for cleavage at the NS2B-NS3 site prior to the NS2A-NS2B site is peculiar to the construct used in our *in vitro* studies or reflects the situation for processing of the intact polyprotein *in vivo*. Differences in cleavage site preferences have been well documented in cleavages catalyzed by the picornavirus 3C (31) and the alphavirus nsP2 (32) proteinases. In both cases, substrate preferences are different when the protease domain is part of larger polyprotein precursors and such differences appear to be important for regulating various steps in virus replication. Further studies will be necessary to determine whether similar mechanisms play a role in altering flavivirus proteinase activity during the course of infection that in turn result in the modulation of RNA synthesis or virion assembly.

Thus far, it has not been possible to clearly demonstrate cleavage at YF NS3-NS4A and NS4B-NS5 sites *in vitro* despite analysis of a number of different constructs in the presence or absence of microsomal membranes. Correct processing at the NS3-NS4A and NS4B-NS5 sites may depend on a specific membrane configuration of the polyprotein or on higher concentrations of protease that were not achieved with the present *in vitro* system. However, in transient expression experiments, we have recently obtained evidence that the NS3 protease also catalyzes these cleavages. Studies aimed at defining the order, kinetics, and cis–trans properties of the viral protease toward these and altered cleavage sites and the importance of these cleavages for virus replication are now in order.

We thank our colleagues for helpful suggestions on the manuscript, Dr. Chang Hahn for help with the figures, Dr. Joel Dalrymple for providing mouse hyperimmune antiserum to YF, and Dr. James Krause for ^{3}H-labeled amino acids. This work was supported in part by a grant from the Pew Memorial Trust and U.S. Army Contract DAMD17-87-C-7154. C.M.R. is a Pew Scholar in the Biomedical Sciences. R.J.F. and J.F.B. were supported by National Institutes of Health Grant DK39304. J.F.B. acknowledges a fellowship from the A. P. Sloan Foundation. T.J.C. was supported in part by National Institutes of Health Grants AI07172, AI07739, and AI00973 and a grant from the American Philosophical Society.

1. Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L. & Strauss, J. H. (1985) *Science* **229**, 726–733.
2. Rice, C. M., Strauss, E. G. & Strauss, J. H. (1986) in *The Togaviridae and Flaviviridae*, eds. Schlesinger, S. & Schlesinger, M. J. (Plenum, New York), pp. 279–326.
3. Chambers, T. J., McCourt, D. W. & Rice, C. M. (1990) *Virology* **177**, 159–174.
4. Ruiz-Linares, A., Cahour, A., Despres, P., Girard, M. & Bouloy, M. (1989) *J. Virol.* **63**, 4199–4209.
5. Chambers, T. J., Hahn, C. S., Galler, R. & Rice, C. M. (1990) *Annu. Rev. Microbiol.* **44**, 649–688.
6. Kräusslich, H.-G. & Wimmer, E. (1988) *Annu. Rev. Biochem.* **57**, 701–754.
7. Bazan, J. F. & Fletterick, R. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7872–7876.
8. Bazan, J. F. & Fletterick, R. J. (1989) *Virology* **171**, 637–639.
9. Gorbalenya, A. E., Donchenko, A. P., Koonin, E. V. & Blinov, V. M. (1989) *Nucleic Acids Res.* **17**, 3889–3897.
10. Chambers, T. J., McCourt, D. W. & Rice, C. M. (1989) *Virology* **169**, 100–109.
11. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
12. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
13. Rice, C. M., Grakoui, A., Galler, R. & Chambers, T. J. (1989) *New Biol.* **1**, 285–296.
14. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
15. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
16. Rice, C. M., Aebersold, R., Teplow, D. B., Pata, J., Bell, J. R., Vorndam, A. V., Trent, D. W., Brandriss, M. W., Schlesinger, J. J. & Strauss, J. H. (1986) *Virology* **151**, 1–9.
17. Palmenberg, A. C. & Rueckert, R. R. (1982) *J. Virol.* **41**, 244–249.
18. Polgar, L. (1987) in *Hydrolytic Enzymes*, eds. Neuberger, A. & Brocklehurst, K. (Elsevier, Amsterdam), pp. 159–200.
19. Liao, D.-L. & Remington, S. J. (1990) *J. Biol. Chem.* **265**, 6528–6531.
20. Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J. P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L. & Menge, U. (1990) *Nature (London)* **343**, 767–770.
21. Winkler, F. K., D'Arcy, A. & Hunziker, W. (1990) *Nature (London)* **343**, 771–774.
22. Carter, P. & Wells, J. A. (1988) *Nature (London)* **332**, 564–568.
23. Higaki, J. N., Evnin, L. B. & Craik, C. S. (1989) *Biochemistry* **28**, 9256–9263.
24. Hahn, C. S. & Strauss, J. H. (1990) *J. Virol.* **64**, 3069–3073.
25. Dougherty, W. G., Parks, T. D., Cary, S. M., Bazan, J. F. & Fletterick, R. J. (1989) *Virology* **172**, 302–310.
26. Ivanoff, L. A., Towatari, T., Ray, J., Korant, B. D. & Petteway, S. R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5392–5396.
27. Cheah, K.-C., Leong, L. E.-C. & Porter, A. G. (1990) *J. Biol. Chem.* **265**, 7180–7187.
28. Graf, L., Craik, C. S., Patthy, A., Roczniak, S., Fletterick, R. J. & Rutter, W. J. (1987) *Biochemistry* **26**, 2616–2623.
29. Craik, C. S., Largman, C., Fletcher, T., Roczniak, S., Barr, P. J., Fletterick, R. & Rutter, W. J. (1985) *Science* **228**, 291–297.
30. Nowak, T., Farber, P. M., Wengler, G. & Wengler, G. (1989) *Virology* **169**, 365–376.
31. Ypma-Wong, M. F. & Semler, B. L. (1987) *Nucleic Acids Res.* **15**, 2069–2088.
32. de Groot, R. J., Hardy, W. R., Shirako, Y. & Strauss, J. H. (1990) *EMBO J.* **9**, 2631–2638.