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Mutagenesis of the Yellow Fever Virus NS2B Protein: Effects on Proteolytic Processing, NS2B-NS3 Complex Formation, and Viral Replication

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To study the role of specific regions of the yellow fever virus NS2B protein in proteolytic processing and association with the NS3 proteinase domain, a series of mutations were created in the hydrophobic regions and in a central conserved hydrophilic region proposed as a domain important for NS2B function. The effects of these mutations on *cis* cleavage at the 2B/3 cleavage site and on processing at other consensus cleavage sites for the NS3 proteinase in the nonstructural region were then characterized by cell-free translation and transient expression in BHK cells. Association between NS2B and the NS3 proteinase domain and the effects of mutations on complex formation were investigated by nondenaturing immunoprecipitation of these proteins expressed in infected cells, by cell-free translation, or by recombinant vaccinia viruses. Mutations within the hydrophobic regions had subtle effects on proteolytic processing, whereas mutations within the conserved domain dramatically reduced cleavage efficiency or abolished all cleavages. The conserved domain of NS2B is also implicated in formation of an NS2B-NS3 complex on the basis of the ability of mutations in this region to eliminate both association of these two proteins and *trans*-cleavage activity. In addition, mutations which either eliminated proteolytic processing or had no apparent effect on processing were found to abolish recovery of infectious virus following RNA transfection. These results suggest that the conserved region of NS2B is a domain essential for the function of the NS3 proteinase. Hydrophobic regions of NS2B whose structural integrity may not be essential for proteolytic processing may have additional functions during viral replication.

Yellow fever virus (YF), the prototype member of the *Flavivirus* genus, contains a single positive-stranded RNA genome which encodes a single long open reading frame believed to generate a polyprotein of over 350 kDa (7, 29, 30). The gene order for the polyprotein has been established as 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′, where C, prM, and E denote structural protein precursors and NS1 through NS5 indicate the nonstructural proteins. Recent investigations have established that several cleavages on the flavivirus polyprotein are mediated by a viral proteinase encoded within the N terminus of the NS3 protein (6, 13, 27, 33, 35). For YF, these cleavage sites include the consensus sites [G(A)RR↓S] which generate the N termini of NS2B, NS3, NS4A, and NS5 (see reference 22, and for a compilation, see reference 7), as well as additional cleavage sites within the NS2A and NS4A regions which have been proposed to occur at QK↓T and QR↓S sites, respectively (21, 26). In addition, the proteinase is believed to mediate a cleavage producing the C terminus of the virion capsid at a proposed consensus site (1). The flavivirus NS3 proteinase contains a serine proteinase domain with homology to the small cellular proteinases of the trypsin superfamily (3, 4, 18). The importance of residues in the proposed serine proteinase catalytic triad (YF, His-Asp-Ser) has been demonstrated by several studies employing site-directed mutagenesis of these residues (2, 11, 33, 35). It has also been established that in addition to an intact proteinase domain, the small nonstructural protein NS2B is required for site-specific cleavage activity at the consensus and additional sites (2, 6, 13, 21, 27, 35). Although the precise function of YF NS2B in processing of the flavivirus polyprotein is not known, several lines of evidence suggest that this protein may associate with NS3 such that the active form of the proteinase is a heterodimer. This evidence includes (i) the observation that cleavage of $2A_{110}$-3_{181} polyproteins to generate NS2B and 3_{181} in vitro is dilution insensitive (11); (ii) the observation that NS2B and the NS3 proteinase domain are both necessary for *trans* cleavage at the 2A/2B, 2B/3, 4A/2K, 4B/5, and anchored capsid cleavage sites (1, 6, 21); and (iii) the recent demonstration that the dengue virus type 2 (DEN2) NS2B and NS3 proteins can be coimmunoprecipitated (2). Without crystallographic data on the structure of the active NS2B-NS3 proteinase, the nature of the interaction between these two proteins remains unknown. However, sequence comparison of flavivirus NS2B proteins reveals a highly conserved and charged domain flanked by regions of hydrophobic amino acids (12). The highly conserved domain has been shown to be essential for NS3-mediated cleavage at the dengue virus type 4 (DEN4) 2B/3 cleavage site (12). Because the predicted topology of the YF polyprotein suggests that processing occurs in association with the membranes of the endoplasmic reticulum, it has been hypothesized that the small hydrophobic proteins NS2A, NS2B, NS4A, and NS4B may be required for association of the large nonstructural proteins with membrane sites (30). Although most of the flavivirus nonstructural proteins have been identified in membrane fractions of infected cells (34), specific regions of these proteins which participate in membrane-protein and protein-protein interactions have not been determined. In this study, several mutations were created within putative cytoplasmic and membrane-associated domains of the YF NS2B protein, and their effects on polyprotein processing, association between NS2B and NS3, cleavage activity of the proteinase in *trans*, and recovery of infectious virus were determined.

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MATERIALS AND METHODS

Cells and viruses. Infection with YF 17D was as described previously (8). Growth of BHK-21, SW-13, BSC-40, and BHK-21-15 cells has been described previously (6, 9). Preparation of vTF7-3, the vaccinia virus expressing T7 RNA polymerase (15), has been described previously (6). CV-1 cells were grown in Earle's minimal essential medium (MEM) containing 5% fetal calf serum.

T7 transcription plasmids. The use of pET plasmids (F. W. Studier, Brookhaven National Laboratories) (32) and pTM3 plasmids (B. Moss, National Institute of Allergy and Infectious Diseases) (25) for cloning and expression of YF proteins under the control of the T7 promoter has been previously described (6, 11). Plasmids were constructed with standard methods and reagents (31), and the structures were verified by restriction enzyme digests and nucleotide sequence analysis for all constructs engineered by oligonucleotide-directed mutagenesis or polymerase chain reaction amplification.

Construction of mutants and site-directed mutagenesis. Mutations within the YF NS2B protein were created by the use of natural restriction sites or by using restriction sites engineered by oligonucleotide-directed mutagenesis. CD38 was constructed by creating two plasmids: pET8c-NS2A$_{110}$-3$_{298}$D *Nco*I, containing a deletion of the coding sequence between the *Nco*I sites at 3834 and 4457 (YF nucleotide numbering), and pET8c-NS2A$_{110}$-3$_{298}$D *Hin*dIII, containing a deletion of the coding sequence between the *Hin*dIII sites at 4343 (YF nucleotide) and 1883 (plasmid sequence). Following digestion with *Nco*I and *Hin*dIII, respectively, the linearized plasmids were filled in with the DNA polymerase I large fragment (Klenow) (*Nco*I site) or digested with mung bean nuclease (*Hin*dIII site), and appropriate fragments were isolated and ligated by blunt-end ligation. The NS2B fragment containing the mutation was subcloned into the plasmid pTM3-NS2B by polymerase chain reaction amplification or introduced into sig2A-5$_{356}$ by exchange of the 770-bp *Sst*I fragment. Mutants CD3 and CD5 were constructed by creation of intermediate pET8c-2A$_{110}$-3$_{181}$ plasmids containing deletions between the *Sst*I sites at 4338 and 5112 (YF nucleotides) and between the *Hin*dIII sites at 4343 (YF nucleotides) and 1833 (plasmid sequence), respectively. Fragments containing filled-in *Sst*I and *Hin*dIII sites were ligated with a three-piece ligation with a vector-derived *Eco*RI-*Ava*I fragment. A few resulting clones unexpectedly contained an *Nhe*I site at NS2B nucleotide 4330. This site was utilized to create mutation CD5 by linearization with *Nhe*I and digestion with mung bean nuclease and religation and also to create mutation CD3 by ligating fragments containing filled-in *Nhe*I and *Hin*dIII sites. For creation of 1D3 and 1I3, two pET/BS(+) 2B-3$_{181}$ plasmids (11) containing *Stu*I sites at NS2B positions 4225 (1.1) and 4234 (1.2) were engineered by site-directed mutagenesis. Constructs containing either a 3-amino-acid insertion (1I3) or a deletion of the same size (1D3) were generated by ligation of *Stu*I-*Ava*I fragments containing the 5′ *Stu*I site of 1.2 and the 3′ *Stu*I site of 1.1 or the 5′ *Stu*I site of 1.1 and the 3′ *Stu*I site of 1.2, respectively. For creation of 2D4, 2I4, and 2D13, two pET/BS(+) 2B-3$_{181}$ plasmids containing an *Eco*RV site at 4273 (2.1) and a *Stu*I site at 4285 (2.2) were engineered by site-directed mutagenesis. A construct containing a 4-amino-acid insertion (2I4) was then generated by ligation of an *Ava*I-*Stu*I fragment containing the 3′ *Stu*I site of 2.2 with an *Ava*I-*Eco*RV fragment containing the 5′ *Eco*RV site of 2.1. A construct containing a 4-amino-acid deletion (2D4) was created by ligating an *Ava*I-*Eco*RV fragment containing the 3′ *Eco*RV site of 2.1 with an *Ava*I-*Stu*I fragment containing the 5′ *Stu*I site of 2.2. Construction of 2D13 was accomplished by ligation of the *Ava*I-*Stu*I fragments containing the 3′ *Stu*I site of 1.2 and the 5′ *Eco*RV site of 2.1. For creation of mutations 3D3 and 3I3, two pET/BS(+) 2B-3$_{181}$ plasmids containing *Stu*I sites at NS2B positions 4475 (3.1) and 4485 (3.2) were engineered by site-directed mutagenesis. Constructs containing either a 3-amino-acid insertion (3I3) or a deletion of the same size (3D3) were then generated by ligation of *Stu*I-*Ava*I fragments containing the 5′ *Stu*I site of 3.1 and the 3′ *Stu*I site of 3.2 or the 3′ *Stu*I site of 3.1 and the 5′ *Stu*I site of 3.2, respectively. For creation of the CD4 mutation, two pET/BS(+) 2B-3$_{181}$ plasmids containing an *Eco*RV site at NS2B 4376 (C4.1) and a *Sca*I site at NS2B 4388 (C4.2) were engineered by site-directed mutagenesis. A construct containing a 4-amino-acid deletion was then generated by ligating an *Ava*I-*Eco*RV fragment with an *Ava*I-*Sca*I fragment. For creation of mutations 1D3D, 1D3I, 1I3D, and 1I3I, the 770-bp *Sst*I fragments of 2B-3$_{181}$ constructs containing the 3D3 and 3I3 mutations were each ligated into each of the 2B-3$_{181}$ plasmid vectors containing the 1D3 and 1I3 mutations after digestion with *Sst*I and dephosphorylation. For construction of sig2A-5$_{356}$ (6) containing the CD38, CD4, 3D3, and 3I3 mutations, the 770-bp *Sst*I fragments from the mutant 2B-3$_{181}$ constructs was ligated into sig2A-5$_{356}$ after digestion with *Sst*I and dephosphorylation. For construction of sig2A-5$_{356}$ plasmids containing the 1D3, 1I3, 2D4, 2D13, 2I3, CD3, and CD5 mutations, sig2A-3$_{181}$ constructs containing these mutations were generated by subcloning *Pvu*II fragments from the mutant 2B-3$_{181}$ constructs into sig2A-3$_{181}$. The mutations were then reconstructed in sig2A-5$_{356}$ by ligation of *Xba*I-*Hpa*I fragments from the sig2A-3$_{181}$ constructs into sig2A-5$_{356}$ after digestion with *Xba*I and *Hpa*I and dephosphorylation. Construction of sig2A-5$_{356}$ plasmids containing the 1D3D and 1I3I mutations was achieved by ligation of the *Sst*I fragments from sig2A-5$_{356}$ constructs containing the 3D3 and 3I3 mutations into sig2A-5$_{356}$ plasmids containing the 1D3 and 1I3 mutations, respectively, after these were digested with *Sst*I and dephosphorylated.

Vaccinia virus recombinants. Plasmids for homologous recombination were generated by cloning the 2B, 3$_{181}$, or 2B-3$_{181}$ regions into pTM3. For pTM3-NS2B, pET8c-NS2B DNA was digested with *Bam*HI and partially with *Nco*I, and the 403-bp fragment was ligated into pTM3 which had been digested with *Bam*HI and *Nco*I. Construction of pTM3-3$_{181}$ was achieved by digestion of pET8c-3$_{181}$ DNA with *Msp*I and filling in the overhanging ends with DNA polymerase I, followed by further digestion with *Nco*I. The 622-bp fragment was then ligated to pTM3 DNA after digestion with *Sma*I and *Nco*I. Construction of pTM3-NS2B-3$_{181}$ was accomplished by digesting pET8c-NS2B-3$_{181}$ with *Nhe*I and partially with *Nco*I. The 1,236-bp fragment was ligated into pTM3 after digestion with *Nco*I and *Spe*I. pTM3-NS2B clones containing mutations CD3, CD5, and CD38 were generated from pET8c-NS2B constructs containing these mutations by the same strategy described for pTM3-NS2B. Vaccinia virus recombinants were generated by infecting CV-1 monolayers with wild-type (wt) vaccinia virus and transfecting with the calcium phosphate-precipitated pTM3-YF constructs (24). Recombinant virus was selected by passaging on human TK$^-$ 143 monolayers in the presence of 25 μg of 5-bromodeoxyuridine per ml. The resulting thymidine kinase-negative vaccinia virus was plaque purified at least twice under *gpt* selection (14) on BSC-40 cells. Large-scale preparations of recombinant virus were grown and titered on BSC-40 or BHK-21 cells in the absence of selection.

In vitro transcription. 5′ capped transcripts were produced from linearized pET8c-YF plasmid DNA templates with T7

RNA polymerase (Epicenter) and conditions recommended by the manufacturer.

Cell-free translation. Cell-free translation was performed with rabbit reticulocyte lysates programmed with pET8c transcripts in accordance with the manufacturer's (Promega) specifications. Proteins were radiolabelled by inclusion of [^{35}S]methionine (Amersham).

Transient expression assay. Expression of YF polyproteins with the transient expression assay has been described previously (6). Briefly, confluent monolayers of BHK-21-15 cells were infected with vTF7-3 (multiplicity of infection = 10); infection was followed by DNA transfection with lipofectin (Bethesda Research Laboratories) (15 μg of lipid per μg of DNA). Following an interval of 2.5 h, cells were labelled with [^{35}S]methionine as previously described (6).

Preparation of infected cell extracts. Extracts of YF-infected BHK-21 cells for preparation of YF-specific proteins were prepared as previously described (9).

Immunoprecipitation. Lysates from transient expression experiments or proteins prepared by cell-free translation were prepared with denaturing conditions as described previously (9, 11). Lysates for analysis of proteinase complex formation were prepared with nondenaturing conditions (1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 1.0% deoxycholate, 150 mM NaCl, 50 mM Tris-Cl [pH 7.5] [buffer A]). Following solubilization of cell monolayers in this detergent mixture, the lysate was centrifuged at 30,000 rpm in an SW50.1 rotor for 30 min at 4°C. The supernatant was removed, and immunoprecipitation was carried out by addition of antiserum to NS2B or NS3. Immunoprecipitates were collected with *Staphylococcus aureus* Cowan strain I (Calbiochem) and then washed three times with buffer A and once with 1 M NaCl–50 mM Tris-Cl (pH 7.5).

Gel electrophoresis. Washed immunoprecipitates were solubilized in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) by methods previously described (20).

Construction of full-length YF cDNA templates and RNA transcription and transfection. pET-8c-sig2A-5_{356} plasmids containing NS2B mutations were used to reconstruct full-length YF cDNA templates by methods previously described (28) with slight modifications. The *Avr*II-*Nhe*I fragment from sig2A-5_{356} clones containing the relevant NS2B mutations was transferred to the YFM 5.2 plasmid, and the *Aat*II-*Nsi*I fragments of the YFM 5.2 clones and YF 5′3′IV were ligated after recovery of appropriate fragments from low-melting-temperature agarose gels. By this method, approximately 100 ng of full-length template was generated on the basis of analysis by agarose gel electrophoresis. Full-length templates were linearized with *Xho*I, and SP6 transcripts were synthesized as previously described (28). Integrity of YF transcripts was determined by nondenaturing RNA gel electrophoresis. Transfection of subconfluent SW-13 cells was carried out with conditions described for BHK-21 cells (28). Briefly, monolayers were washed with phosphate-buffered saline (PBS) and transfected with a mixture of PBS, lipofectin (Bethesda Research Laboratories), and RNA transcripts for 10 min at room temperature. The transfection mixture was then removed, cells were washed with MEM, and then the monolayer was overlaid with 1% agarose–MEM containing 2% fetal calf serum–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.0. Plaques were visualized after 96 h by staining with neutral red or crystal violet. For recovery of virus for plaque assay, monolayers transfected with 250 ng of transcripts were incubated in MEM containing 2% fetal calf serum for 60 to 72 h, following which the medium was removed, clarified by low-speed centrifugation, and stored at −70°C until the plaque assay was performed. Virus was titered by plaque assay on SW-13 cells with overlay conditions as described above.

RESULTS

Structures of NS2B mutations. Figure 1 illustrates the YF NS2B protein with the conserved central 40-amino-acid domain flanked by three regions of high hydrophobicity. Since the 2A/2B and 2B/3 cleavages are presumed to occur in the cytoplasm, the predicted topology of the polyprotein suggests that these hydrophobic regions may be membrane-associated segments and the conserved region may be a cytoplasmic domain, although this has not been proven. The three hydrophobic regions and the conserved domain were targeted by mutations consisting of in-frame deletions or insertions, in some cases accompanied by conservative or nonconservative amino acid substitutions. Mutation 1D3 contains a 3-amino-acid deletion of residues 18 to 20 (relative to the NS2B N terminus) associated with the substitution of G for V at residue 16. The 1I3 mutation contains a repeat of residues 18 to 20 (AGL) inserted between residues 17 and 18. The 2D4 mutation contains a 4-amino-acid deletion of residues 33 to 36 (AVGG). The 2I4 mutation consists of a 4-amino-acid insertion (VGGS) between residues 33 and 34, associated with the substitution of S for A at residue 33. The 2D13 deletion removes residues from positions 21 to 33, associated with the substitution of S for L at residue 21. Mutations CD3 and CD5 in the conserved domain consist of 3- and 5-amino-acid deletions (LEL and LELKK) from residues 53 to 55 and 51 to 55, respectively. CD38 consists of a deletion of 38 amino acids from residues 56 to 93, associated with the substitution of T for P at residue 93. CD4 consists of a 4-amino-acid deletion (ISGS) from residues 67 to 70, associated with the conservative substitutions of D for E at residue 66 and T for S at residue 71. The 3D3 mutation consists of a deletion of three residues (TSL) from residues 100 to 102, associated with the nonconservative substitution of K for M at residue 99. 3I3 contains an insertion of 3 amino acids (SQL) between residues 100 and 101. The 1D3D and 1I3I double mutations consist of combinations of 1D3 with 3D3 and 1I3 with 3I3, respectively.

Cell-free translation of 2B-3_{181} transcripts containing NS2B mutations. Figure 2 illustrates the cleavage patterns of 2B-3_{181} polyproteins containing the mutations in the hydrophobic and conserved domains of NS2B. Following 1 h of translation, the wt 2B-3_{181} polyprotein is cleaved almost completely to the 21-kDa 3_{181} and 14-kDa NS2B proteins (lane b). 2B-3_{181} polyproteins containing mutations in any of the three hydrophobic regions exhibited levels of cleavage similar to that of the wt (lanes c to g and l to q), although precise quantitation of these cleavage reactions was not performed. In these experiments, cleavage efficiency is based on the amount of the 21-kDa 3_{181} protein formed, because of differences among mutant forms of NS2B (see below). The mutant NS2B proteins exhibited electrophoretic mobilities consistent with their predicted molecular weights (Fig. 1). Mutant forms of NS2B containing deletions within the first hydrophobic region (1D3, 2D13, 1D3D, and 1D3I, Fig. 2, lanes c, e, n, and o) were present in diminished amounts compared with the wt form. Such deletions may render the NS2B protein more susceptible to degradation in the reticulocyte lysate. However, these mutations may alter the reactivity of NS2B with the antisera used for immunoprecipitation. In the absence of immunoprecipitation, comigration of NS2B with endogenously produced globin obscures any ability to differentiate these possibilities (data not shown). In contrast to mutations in the hydrophobic

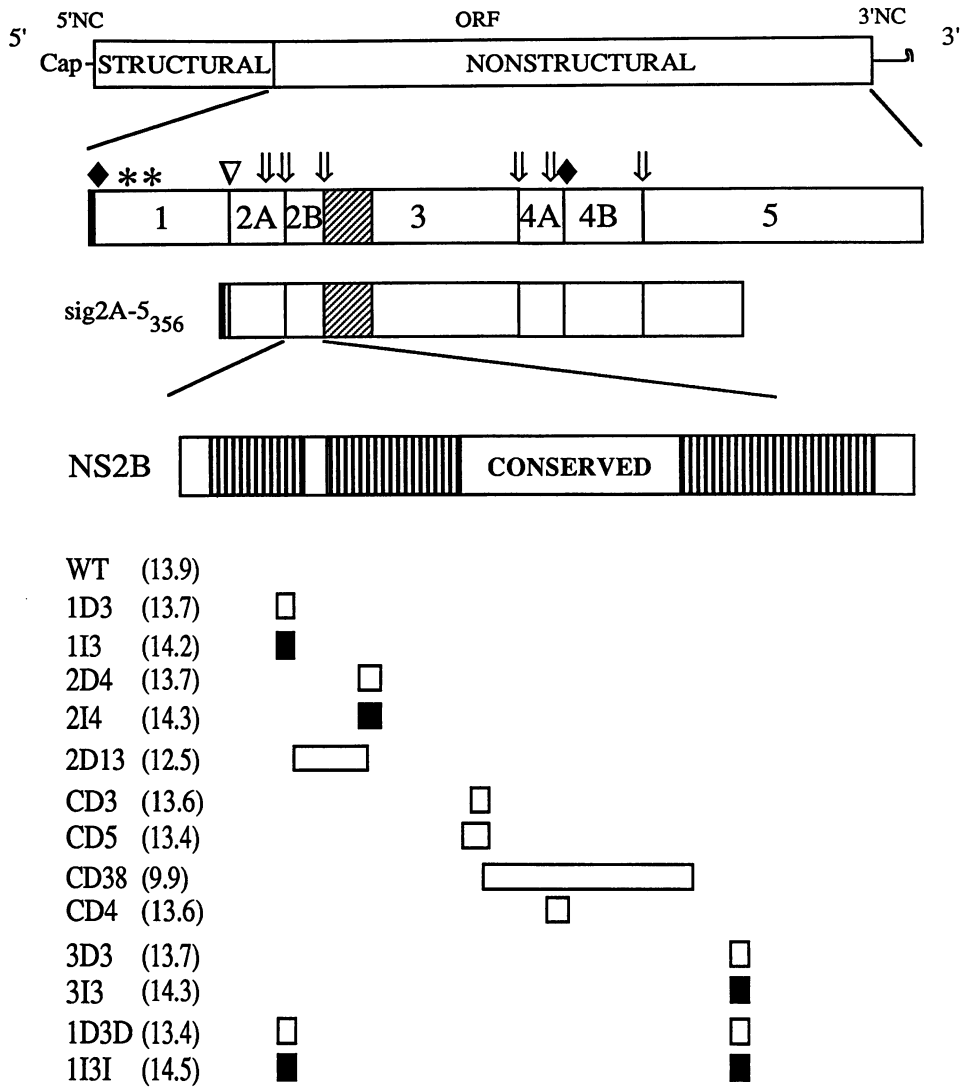

FIG. 1. Schematic of the YF genome, nonstructural polyprotein, the sig2A-5_{356} construct, and the NS2B protein with the mutations engineered within this protein. The sig2A-5_{356} construct (6) contains the signal sequence (solid region) preceding NS1 fused to NS2A and extends through the first 356 amino acids of NS5. Asterisks indicate potential N-linked glycosylation sites. The shaded region indicates the NS3 proteinase domain. Putative signalase cleavages are represented by solid diamonds. Open arrows indicate cleavages mediated by NS3 proteinase. The open inverted triangle indicates NS1-2A cleavage by an undefined proteinase. Within the NS2B protein, the central conserved domain is indicated. Vertical striped regions indicate the positions of the hydrophobic regions. Mutations are indicated by open boxes (deletions) or solid boxes (insertions). The predicted molecular weights (in thousands) of NS2B proteins containing these mutations are indicated in parentheses. ORF, open reading frame.

domains, 2B-3_{181} polyproteins containing deletions in the conserved domain (CD3, CD38, CD4, and CD5, lanes h to k) exhibited no cleavage products consistent in size with 3_{181} or NS2B. A minor amount of NS2B and 3_{181} was detectable from the CD4 mutant after prolonged exposure (data not shown). The relative amount of the 2B-3_{181} polyprotein containing the CD38 deletion was diminished. Although this deletion reduces immunoreactivity with NS2B antisera (data not shown), reactivity with antisera to NS3 should not be altered in the presence of SDS. This suggests that the CD38 deletion renders the 2B-3_{181} polyprotein unstable in this expression system. Taken together, these results suggest that the conserved domain within NS2B is necessary for site-specific cleavage at the 2B/3 cleavage site.

Effect of mutations in NS2B on processing at the 2A/2B, 3/4A, 4A/4B, and 4B/5 cleavage sites. In order to determine whether mutations within NS2B which permit or abolish cleavage at the 2B/3 cleavage site in cell-free translation alter the NS3 proteinase-mediated cleavages at additional nonstructural cleavage sites, processing of sig2A-5_{356} polyproteins containing selected NS2B mutations was analyzed with transient expression in BHK-21-15 cells (6). Figure 3A illustrates the patterns of cleavages from mutants 1D3, 1I3, 3D3, and 3I3 analyzed by antisera to NS2B. Compared with processing of the wt polyprotein (lane d), all of these mutant polyproteins exhibited essentially similar patterns of processing (lanes e to h), although the apparent molecular weights of the 2B-specific proteins are altered because of the presence of deletions or

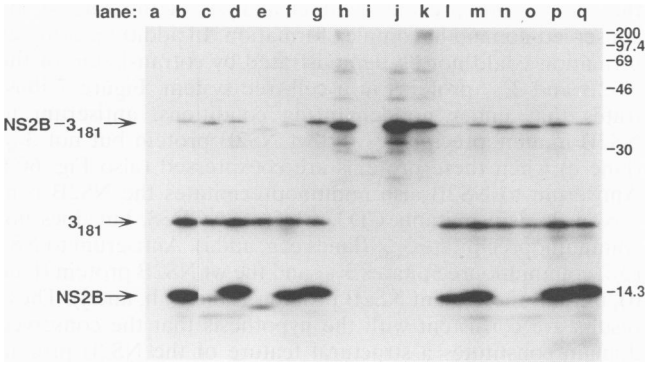

FIG. 2. Cell-free cleavage activity of 2B-3_{181} polyproteins containing mutations in NS2B. T7 RNA transcripts were used to program rabbit reticulocyte lysate, and incubations were carried out for 60 min at 30°C. Following RNase treatment, samples were treated with SDS (0.5%), heated to 70°C for 5 min, and then diluted and immunoprecipitated as described in Materials and Methods with a mixture of antisera specific for the YF NS2B and NS3 proteins. Immunoprecipitated proteins were analyzed by electrophoresis on SDS–15% polyacrylamide gels, and proteins were visualized by fluorography with sodium salicylate. Lanes: a, no RNA; b, wt; c, 1D3; d, 1I3; e, 2D13; f, 2D4; g, 2I4; h, CD3; i, CD38; j, CD4; k, CD5; l, 3D3; m, 3I3; n, 1D3D; o, 1D3I; p, 1I3D; q, 1I3I. Molecular weights, in thousands, are indicated at right.

insertions. Figure 3B illustrates the processing of these mutant polyproteins as analyzed with antisera to the NS3 and NS4B proteins. The sig2A-5_{356} polyproteins containing NS2B mutations 1D3, 1I3, and 3I3 (lanes d and j, e and k, and g and m) exhibited patterns of NS3- and NS4B-specific cleavage products similar to those generated by the wt polyprotein (lanes c and i). The NS3-specific proteins include the 75-kDa NS3 protein and a more slowly migrating form (80 kDa) consistent with an uncleaved NS3-4A protein. The NS3-4A species has been variably observed in this assay and in YF-infected cells and is believed to result from inefficient cleavage at the 3/4A cleavage site (6). In contrast, the 3D3 mutation (lanes f and l) generated a reduced amount of the mature NS3 protein (75 kDa) relative to the wt, suggesting that this deletion may further impair cleavage efficiency at the 3/4A cleavage site.

Figure 4 illustrates the effects of conserved domain mutations CD3, CD4, CD5, and CD38 on processing of the sig2A-5_{356} polyprotein, as analyzed with antisera to NS2B, NS3, or NS4B. In contrast to the cleavage products generated by the wt polyprotein (Fig. 4A, lane d, and 4B, lanes d and k), expression of sig2A-5_{356} polyproteins containing mutations CD5, CD3, and CD38 yielded no detectable processing (Fig. 4A, lanes f to h), but only a high-molecular-weight protein which comigrated with the unprocessed polyprotein generated by replacing the putative catalytic NS3 serine residue with alanine (NS3 amino acid position 138) (11) (Fig. 4A, lane 3, and B, lanes e and l). The sig2A-5_{356} polyprotein containing the CD4 deletion generated NS2B-, NS3-, and NS4B-specific proteins which were consistent with processing at the 2A/2B, 2B/3, 4A/4B, and 4B/5 cleavage sites, although at low efficiency (Fig. 4A, lane i, and 4B, lanes i and p). The 3/4A cleavage is apparently very inefficient in the presence of this mutation. The NS2B-specific proteins included a species with an electrophoretic mobility slightly faster than that of the wt NS2A-2B cleavage product, consistent with the 4-amino-acid deletion in NS2B. Small amounts of NS2B-specific proteins migrating with molecular masses slightly smaller than 18 and 14 kDa were also observed after prolonged exposure. These results suggest that the CD4 mutation reduces cleavage efficiency rather than abolishing NS2B-3 proteinase activity as is the case with the other conserved domain mutations.

Figure 5 illustrates the processing patterns of sig2A-5_{356} polyproteins containing the NS2B mutations 2D4, 2I4, 2D13, 1D3D, and 1I3I as analyzed with NS2B-, NS3-, or NS4B-specific antisera. The mutant polyproteins generated NS2B-specific proteins whose molecular weights corresponded to the sizes predicted by the engineered insertions or deletions (Fig. 5A, lanes e to i, and 5B, lanes d to h and k to o). The presence of mutations 2D13 and 1D3D consistently led to reduced levels of NS2B-related proteins in transient expression, and proteins corresponding to mutant forms of NS2B were observed only after long exposures. Results with the NS3 antisera revealed that only the 1D3D mutation had any detectable effect on production of NS3-specific cleavage products, and similar to the 3D3 mutation (Fig. 3B, lane f), the 3/4A cleavage efficiency was reduced. An additional protein of approximately 27 kDa in molecular mass which reacted with NS3-specific antisera was observed with the 2I4 and 1I3I mutations (Fig. 5B, lanes e and h). The nature of this protein was not further characterized. None of these mutations had any detectable effect on the production of NS4B-specific cleavage products.

Evidence for association of NS2B and the NS3 proteinase domain. Previous studies suggested that YF NS2B and NS3 may be associated as a complex (11). That mutations in the conserved domain of NS2B dramatically reduced or abolished processing of the nonstructural polyprotein suggests that this region may be involved in the formation of an NS2B-NS3 complex. A physical association between these two proteins was studied by coimmunoprecipitation experiments using different sources of proteinase components and either NS2B-specific or NS3-specific antisera. Figure 6 illustrates the results of coimmunoprecipitation using samples from YF-infected cells, cell-free translated 2B-3_{181}, or vaccinia virus recombinants expressing the 2B-3_{181} polyprotein or 2B and 3_{181} proteins individually as sources of proteinase. With infected cells, antiserum to NS2B weakly coimmunoprecipitates NS2B and NS3 under nondenaturing conditions (Fig. 6A, lane f), whereas antiserum 3_C coimmunoprecipitates NS2B, NS3, and additional proteins (lane g) which comigrate with NS4B and the two forms of NS2A (8). Antiserum 3_r weakly coimmunoprecipitates NS3 and small quantities of NS2B under these conditions (lane h). With cell-free translated 2B-3_{181} as a source of proteinase components, antiserum 2B, but not 3_r, coimmunoprecipitated both NS2B and NS3 (Fig. 6B, lanes d and f, respectively), whereas antiserum 3_C weakly coimmunoprecipitated NS2B (lane e). Similar to results with infected cells, antisera to NS2B and 3_C coimmunoprecipitated NS2B and 3_{181} when expressed from a vaccinia virus recombinant expressing 2B-3_{181} (Fig. 6C, lanes c and d). Antiserum 3_C, but not 2B, coimmunoprecipitated both NS2B and 3_{181} when these proteins were expressed with separate vaccinia virus recombinants (lanes e and f). These results suggest that differences may exist between the interaction of NS2B and NS3 or 3_{181} in cell-free and cellular systems (see Discussion).

Complex formation between 3_{181} and NS2B proteins containing mutations in the conserved domain. Because results with nondenaturing conditions indicated that NS2B and 3_{181} could be coimmunoprecipitated, the importance of the conserved domain in NS2B for this association was analyzed by coimmunoprecipitation experiments using vaccinia virus recombinants expressing 3_{181} and NS2B proteins containing mutation CD3, CD5, or CD38. This approach was undertaken because lack of cleavage of 2B-3_{181} polyproteins containing

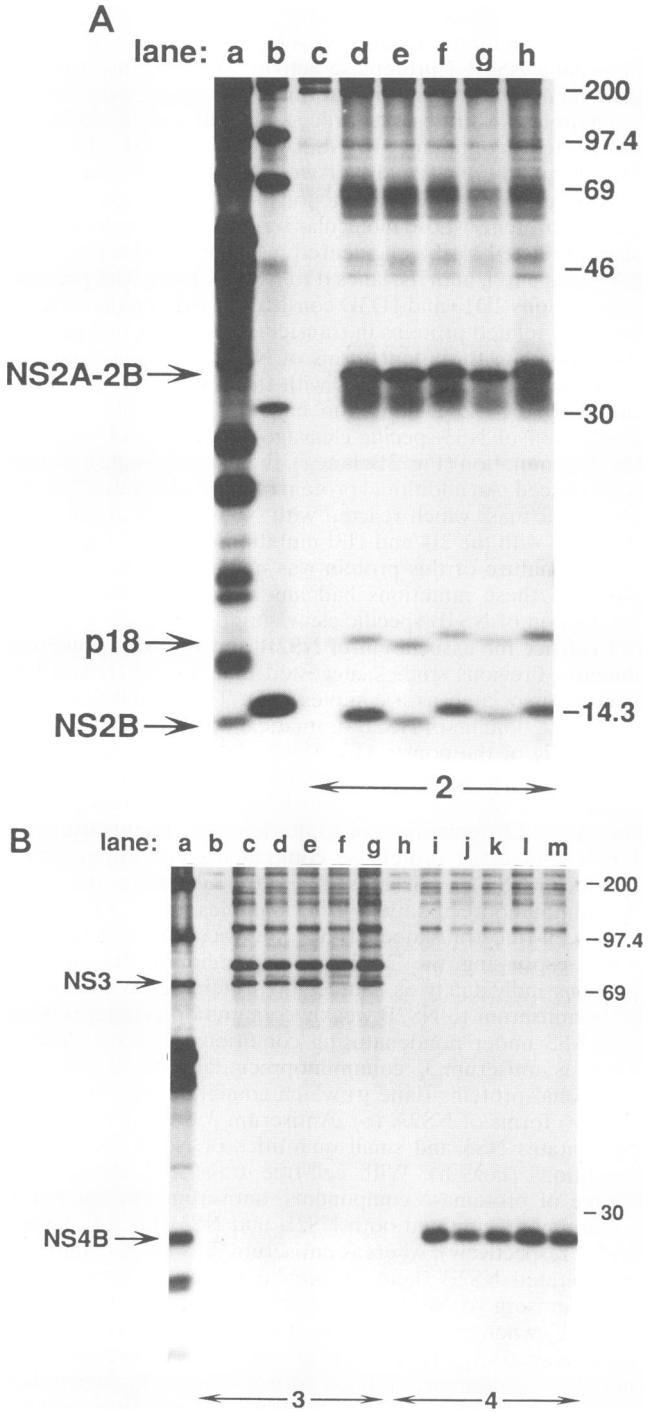

FIG. 3. Transient expression of sig2A-5_{356} polyproteins containing mutations in NS2B. BHK-21-15 cells were infected with vTF7-3 and transfected with pET-8c-YF DNAs as described in Materials and Methods. Cells were labelled for 4.5 h with [^{35}S]methionine, and SDS-solubilized lysates were prepared and immunoprecipitated with antisera to YF nonstructural protein NS2B, -3, or -4B (indicated at bottom). Immunoprecipitates were analyzed by SDS–14% (A) or –12% (B) PAGE, followed by fluorography. The positions of YF proteins from transfections or from YF-infected BHK-21 cells immunoprecipitated with mouse hyperimmune ascitic fluid are indicated at the left. Sizes of molecular weight standards (in thousands), as determined with ^{14}C-labelled high-molecular-weight proteins, are indicated on the right. (A) NS2B-specific cleavage products. Lanes: a, YF proteins; b, high-molecular-weight markers; c, mock-transfected cells (vTF7-3 infection only); d, wt sig2A-5_{356}; e, 1D3; f, 1I3; g, 3D3; h, 3I3. (B) NS3- and NS4B-specific cleavage products. Lanes: a, YF proteins; b and h, mock-transfected cells; c and i, wt sig2A-5_{356}; d and j, 1D3; e and k, 1I3; f and l, 3D3; g and m, 3I3.

these mutations precluded direct analysis of the role of the conserved domain in complex formation. In addition, complex formation could not be demonstrated by cotranslation of the NS2B and 3_{181} proteins in a cell-free system. Figure 7 illustrates that under nondenaturing conditions, antiserum to NS2B immunoprecipitates the wt NS2B protein but not 3_{181} (lane c) when these proteins are coexpressed (also Fig. 6C). Antiserum to NS2B also immunoprecipitates the NS2B conserved domain mutants CD3, CD5, and CD38, but does not coimmunoprecipitate 3_{181} (lanes e, g, and i). Antiserum to NS3 (3_C) coimmunoprecipitates 3_{181} and the wt NS2B protein (lane d), but not the mutant NS2B proteins (lanes f, h, and j). These results are consistent with the hypothesis that the conserved domain constitutes a structural feature of the NS2B protein which is important for the association between NS2B and the NS3 proteinase domain (3_{181}). These coimmunoprecipitation experiments included a 1 M NaCl wash for which the association of 3_{181} with at least some of the wt NS2B protein appears to be stable. It is possible that 3_{181} can associate with the NS2B mutants under less stringent salt conditions such that the deletion of charged residues may reduce but not necessarily eliminate association. In such a case, some functional activity could remain (see below).

Cleavage assay of wt and mutant NS2B proteins expressed in *trans*. In order to determine whether the conserved domain mutations CD3, CD5, and CD38 might still allow functional proteinase activity despite their effects on reducing association between NS2B and 3_{181} under high salt conditions, the ability of these mutants to allow *trans* cleavage in conjunction with 3_{181} (6) was tested. Vaccinia virus recombinants expressing NS2B-3_{181}, NS2B and 3_{181}, or 3_{181} and each of the NS2B mutants CD3, CD5, and CD38 were used as potential sources of proteinase for cleavage of the substrate 3_{540}-5_{356} (6) which contains intact 3/4A, 4A/2K, 4A/4B, and 4B/5 cleavage sites, but no NS3 proteinase domain. Appearance of the NS4B protein is used as evidence of *trans* cleavage at the 4A/2K (21) and 4B/5 sites. Expression of the NS2B-3_{181} recombinant or coexpression of the NS2B and 3_{181} recombinants generated proteinase activity which cleaved the 3_{540}-5_{356} substrate to generate NS4B (Fig. 8, lanes l and m). (Cleavage at the 3/4A site was not examined.) In contrast, coexpression of the 3_{181} recombinant with any of the three recombinant NS2B mutants failed to generate an active proteinase, on the basis of the lack of cleavage of the 3_{540}-5_{356} substrate (lanes n to p). Antisera to NS2B immunoprecipitated proteins corresponding to the wt and mutant NS2B proteins (data not shown). These results suggest that mutations which abolish association of NS2B and the 3_{181} proteinase domain also eliminate *trans*-cleavage activity.

Effect of mutations in NS2B on recovery of infectious virus. In a previous study, it was demonstrated that mutations which reduce or abolish NS3 proteinase activity are deleterious for viral replication (11). Because the NS2B mutations studied here had variable effects on proteolytic processing, their effects on viral replication were therefore determined. The phenotypes of five of these mutations (1D3, 2D4, 3D3, CD3, and CD4) were determined by constructing full-length YF cDNA templates and assaying the infectivity of RNA transcripts

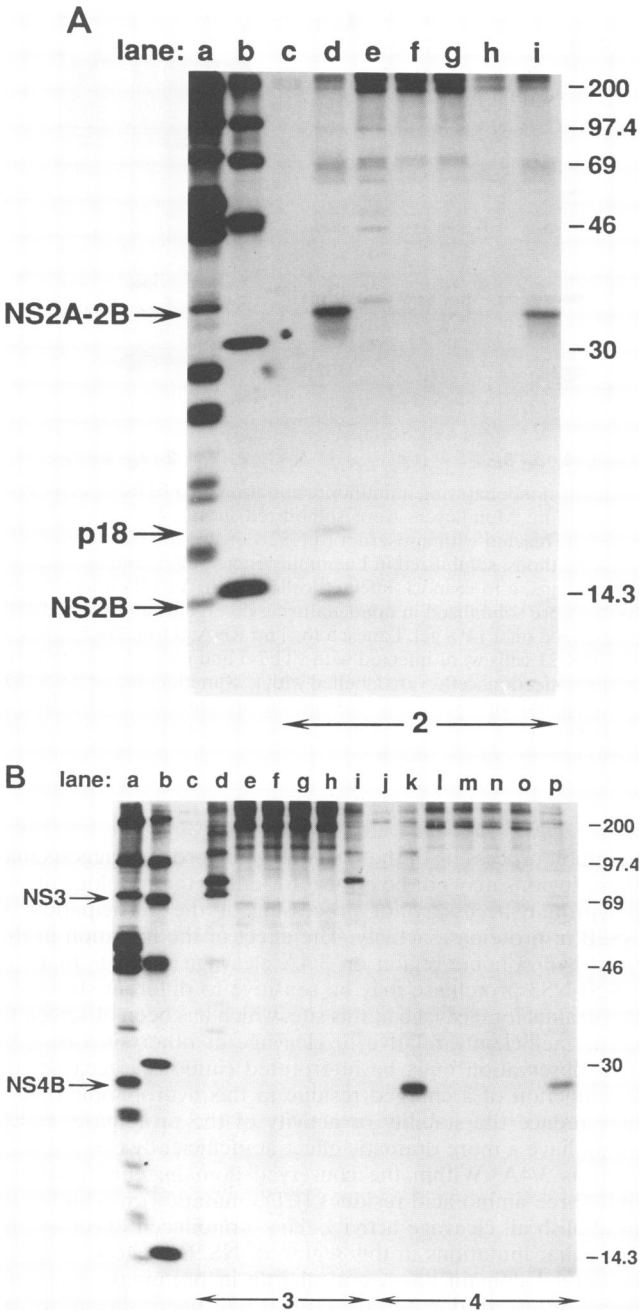

FIG. 4. Transient expression of sig2A-5_{356} polyproteins containing mutations in the NS2B conserved domain. Experimental procedures, YF and molecular weight markers, and figure labels are as described in the legend to Fig. 3. (A) NS2B-specific cleavage products. Lanes: a, YF proteins; b, high-molecular-weight markers; c, mock-transfected cells; d, wt; e, transfection with sig2A-5_{356} containing substitution of alanine for serine at NS3 amino acid 138 (sig2A-5_{356}*); f, CD5; g, CD3; h, CD38; i, CD4. (B) NS3- and NS4B-specific cleavage products. Lanes: a, YF proteins; b, high-molecular-weight markers; c and j, mock-transfected cells; d and k, wt sig2A-5_{356}; e and l, sig2A-5_{356}*; f and m, CD5; g and n, CD3; h and o, CD38; i and p, CD4.

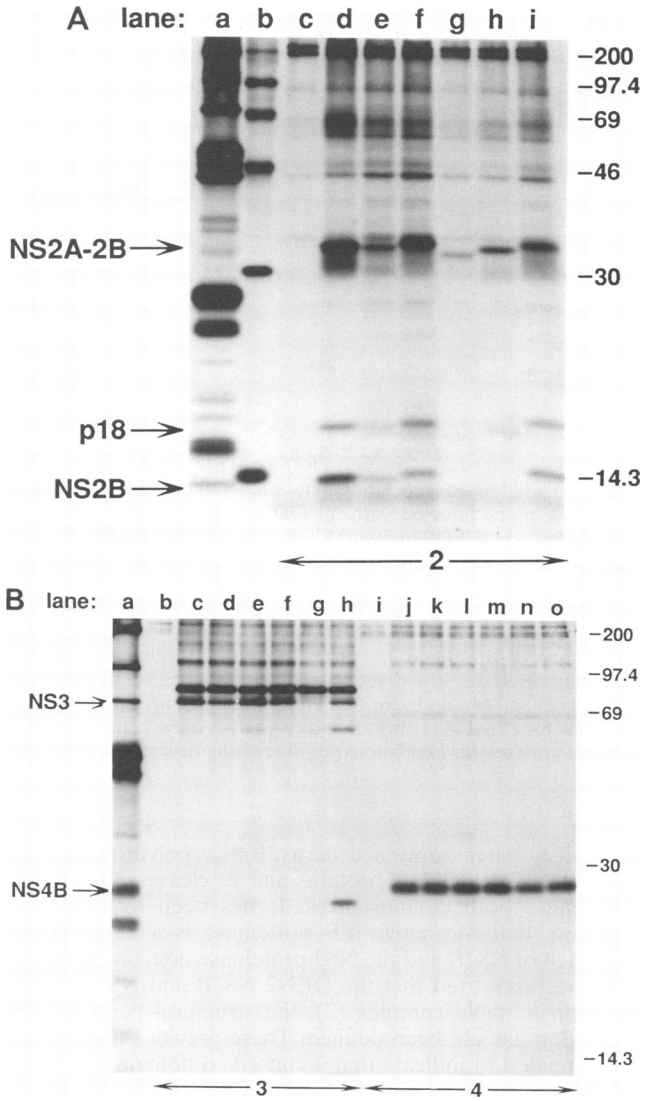

FIG. 5. Transient expression of sig2A-5_{356} polyproteins containing mutations in NS2B. Experimental procedures, YF and molecular weight markers, and figure labels are as described in the legend to Fig. 3. (A) NS2B-specific cleavage products. Lanes: a, YF proteins; b, high-molecular-weight markers; c, mock-transfected cells; d, wt sig2A-5_{356}; e, 2D4; f, 2I4; g, 2D13; h, 1D3D; i, 1I3I. (B) NS3- and NS4B-specific cleavage products. Lanes: a, YF proteins; b and i, mock-transfected cells; c and j, wt sig2A-5_{356}; d and k, 2D4; e and l, 2I4; f and m, 2D13; g and n, 1D3D; h and o, 1I3I.

derived from either wt or mutant constructs. In contrast to wt transcripts, which yielded more than 200 plaques after transfection of 250 ng of RNA, none of the mutant transcripts were capable of generating infectious virus by plaque assay. Medium collected at 72 h after transfection with 250 ng of transcripts was analyzed for infectious virus by plaque assay on SW-13 cells. wt transcripts yielded 10^7 PFU/ml, whereas no infectious virus was recovered after transfection with mutant transcripts (data not shown). These results suggest that mutations in the hydrophobic domains as well as in the conserved domain of NS2B are deleterious for viral replication.

DISCUSSION

In previous studies, the absolute requirement for NS2B for cleavages mediated by the YF NS3 proteinase within the nonstructural region of the polyprotein was demonstrated (6, 21). These two proteins have been shown to generate protein-

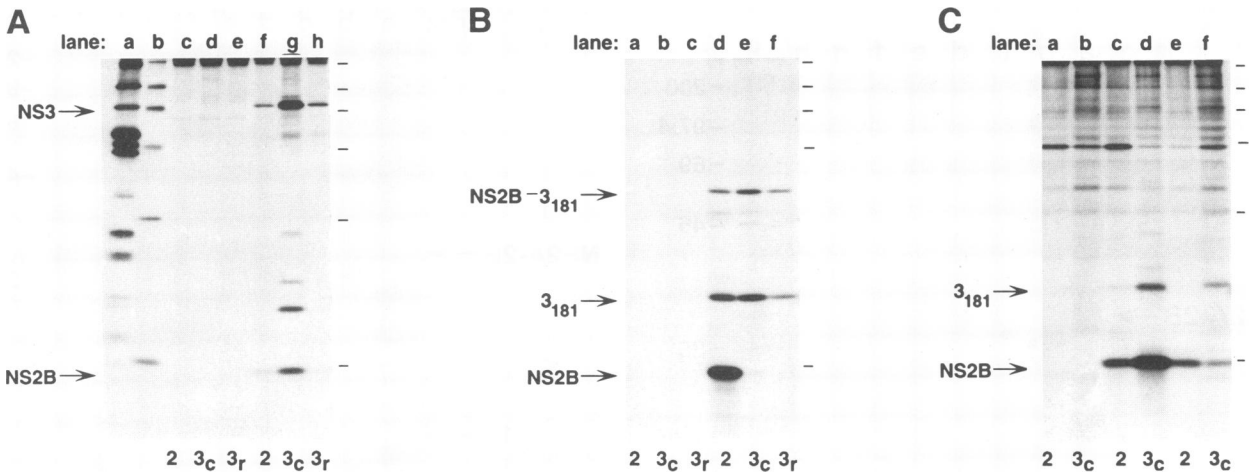

FIG. 6. Analysis of association between NS2B and the NS3 proteinase domain by nondenaturing immunoprecipitation. (A) BHK-21 cells were infected or mock infected with YF17D and labelled from 18 to 24 h postinfection. Monolayers were solubilized in 1% Nonidet P-40–1% deoxycholate–0.1% SDS in 50 mM Tris (pH 7.5)–150 mM NaCl, and the lysate was reacted with antiserum to NS2B or two different antisera to NS3 (3_c and 3_r). Immunoprecipitates were washed as described in Materials and Methods, solubilized in Laemmli sample buffer, and analyzed by SDS-PAGE with a 14% gel. Lanes: a, YF proteins; b, high-molecular-weight proteins; c to e, mock-infected cells; f to h, YF17D infected. (B) Analysis of NS2B and 3_{181} association in cell-free translation. Reticulocyte lysates were solubilized in nondenaturing detergents and reacted with NS2B or NS3 antisera as described above. Immunoprecipitated proteins were analyzed on a 14% gel. Lanes: a to c, no RNA; d to f, 2B-3_{181} RNA. (C) Analysis of NS2B and 3_{181} association using vaccinia virus recombinants. BHK-21 cells were infected with vTF7-3 and either a recombinant expressing NS2B-3_{181} or two recombinants expressing NS2B and $NS3_{181}$. At 3 h postinfection, cells were labelled with [^{35}S]methionine as described in Materials and Methods, for 4 h. Following labelling, immunoprecipitation and analysis of NS2B- and NS3-specific proteins were carried out as described for panel A. Lanes: a and b, vTF7-3 only; c and d, vNS2B-3_{181}; e and f, vNS2B plus $v3_{181}$. Lines to the right of each panel indicate molecular masses (in kilodaltons) in decreasing order: 200, 97.4, 69, 46, 30 (except in panel B), and 14.3.

ase activity when expressed as a 2B-3_{181} polyprotein or as separate NS2B and 3_{181} proteins and to cleave substrates in *trans* under both circumstances. It has been hypothesized, therefore, that the active YF proteinase is a heterodimer, composed of NS2B and the NS3 proteinase domain. Although it has been reported that the DEN2 NS2B and NS3 proteins can form a stable complex (2), the structural basis for this interaction has not been defined. These previous findings are now extended to indicate that a conserved domain within the YF NS2B protein is essential for several properties of the proteinase. These include *cis* cleavage at the 2B/3 cleavage site; *trans* cleavage, at least at the 4B/5 site; and association of NS2B with the NS3 proteinase domain. In addition, the conserved domain and other regions within NS2B are required for YF replication.

The mutations studied in these experiments had variable effects on processing of the YF nonstructural polyprotein precursor. Whereas no major alteration of the pattern of processing was observed with mutations in the first two hydrophobic regions (1D3, 1I3, 2D4, 2D13, 2I4, 3I3, and 1I3I), reduced cleavage efficiency at the 3/4A site was observed when mutations were introduced into the third hydrophobic region (3D3 and 1D3D). Mutations within the conserved domain either reduced cleavage efficiency at all sites (CD4) or abolished all cleavage activity (CD3, CD5, and CD38). Interpreting the role of specific regions of NS2B targeted by these mutations is limited by the fact that certain mutations included either conservative or nonconservative amino acid substitutions. However, a similar approach for analyzing the role of hydrophobic residues in the processing of the poliovirus polyprotein has yielded useful information (16), and in some cases, even nonconservative substitutions have been tolerated in membrane-associated regions of proteins (5). The failure of mutations in the first and second hydrophobic regions of NS2B to inhibit processing of the sig2A-5_{356} polyprotein suggests that these regions may not be critical for either the topology of the polyprotein necessary for processing or the participation of NS2B in proteinase activity. The effect of the mutation in the third hydrophobic region on 3/4A cleavage suggests that the NS2B-NS3 proteinase may be sensitive to different structural constraints for cleavage at this site, which has been observed to occur inefficiently relative to cleavage at other sites (6, 23). This observation must be interpreted cautiously because the introduction of a charged residue in this hydrophobic region may reduce the stability or activity of the proteinase, which might have a more dramatic effect at inefficiently cleaved sites such as 3/4A. Within the conserved domain, elimination of only three amino acid residues (CD3 mutation) was sufficient to abolish all cleavage activity. The pronounced effect of this and other mutations in this region of NS2B suggests that the conserved domain has a critical role in proteinase function. The conserved domain of DEN4 has been shown to be required for *cis* cleavage of NS2B-3 polyproteins and specifically to encompass a 40-amino-acid region whose N and C termini have been precisely determined (12). The N-terminal region of the DEN4 NS2B conserved domain was found to be especially important for cleavage activity, and the results with the CD3 and CD5 mutations reported here are consistent with this finding. In fact, comparison of the homology among flaviviruses reveals a higher level of conservation among residues in the N-terminal portion of the domain (12). Results reported here with the CD4 mutation indicate that certain residues, particularly toward the C-terminal end of the 40-amino-acid domain, may be less critical for proteinase activity. It is unlikely that the effects of mutations in the conserved region result merely from distortion of the configuration of the 2B/3 cleavage site. sig2A-5_{356} polyproteins containing the CD38 mutation can be cleaved in *trans* at the 2B/3 cleavage site

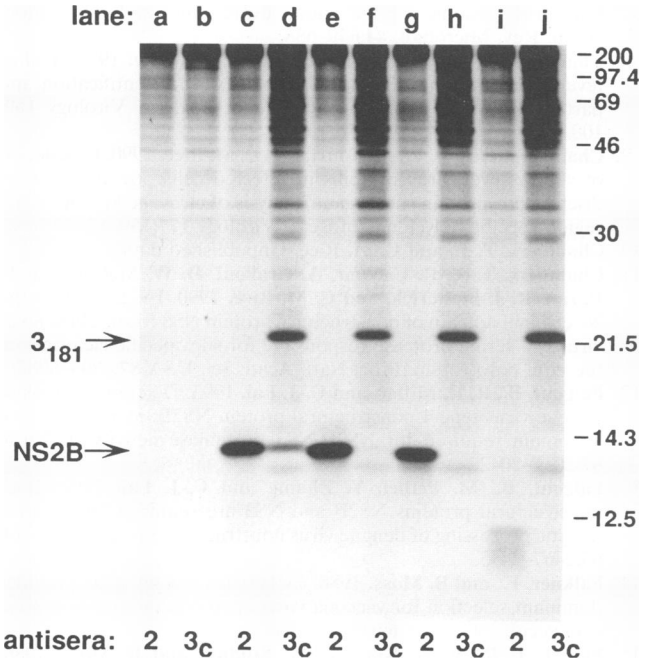

FIG. 7. Analysis of NS2B and NS3 association using vaccinia virus recombinants expressing NS2B deletions in the conserved domain. Experimental procedures were performed as described in the legend to Fig. 6C. Lanes: a and b, vTF7-3 only; c and d, vNS2B plus $v3_{181}$; e and f, vNS2BCD3 plus $v3_{181}$; g and h, vNS2BCD5 plus $v3_{181}$ i and j, vNS2BCD38 plus $v3_{181}$.

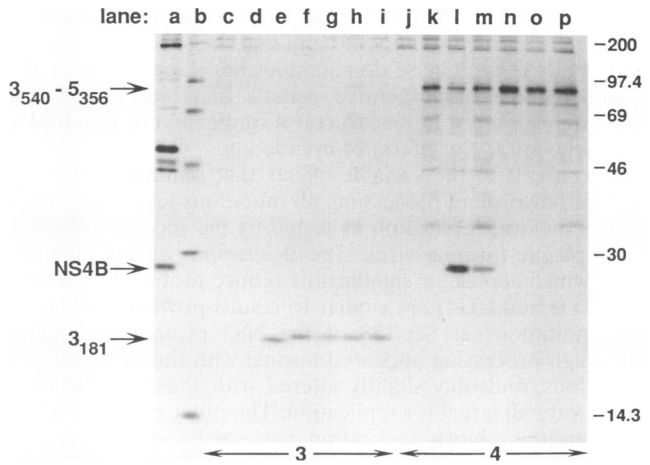

FIG. 8. *trans*-cleavage analysis using 3_{181} and NS2B conserved domain mutants. BHK-21-15 cells were transfected as described in Materials and Methods and in the legend to Fig. 3. Following coinfection with vTF7-3, $v3_{181}$, and vaccinia virus recombinants expressing wt or mutant NS2B proteins, cells were transfected with 3_{540}-5_{356}. Cells were labelled with [^{35}S]methionine for 5 h, following which immunoprecipitation and analysis of proteins were carried out as described in the legend to Fig. 3. Lanes: a, YF proteins; b, high-molecular-weight markers; c and j, vTF7-3 alone; d and k, vTF7-3 plus 3_{540}-5_{356} DNA; e and l, vNS2B-3_{181}; f and m $v3_{181}$ plus vNS2BCD3; g and n, $v3_{181}$ plus vNS2BCD3; h and o, $v3_{181}$ plus vNS2BCD5; i and p, $v3_{181}$ plus vNS2BCD38.

(10), indicating that the cleavage site is still accessible in the context of this mutation. Similar findings have been observed with the DEN4 2B/3 cleavage site (12). Furthermore, mutations which reduce or abolish YF 2B/3 cleavage do not affect processing at other cleavage sites (10), suggesting that interaction of the NS3 proteinase domain with NS2B may precede cleavage at the 2B/3 site and other proteinase-dependent cleavage sites.

The results reported here also suggest that the conserved domain may be an important determinant of the interaction between NS2B and the NS3 proteinase domain. With different systems for expression of NS2B and NS3, these proteins have been shown to remain associated during immunoprecipitation under nondenaturing conditions, although the results depend on the source of the proteins and the antibody used. Differences were observed in the association of NS2B and NS3 among cellular extracts and cell-free translation systems. These findings may result from differences in the folding of the proteins in these two systems, resulting in either a decrease in availability of antibody to specific binding sites or an alteration in the proportions of complexed versus uncomplexed NS2B and NS3. Only a limited fraction of NS2B appears to associate in *trans* with the proteinase domain under nondenaturing conditions. This could reflect the fact that association of the two proteins is inefficient when they are produced in *trans* relative to *cis* because of concentration effects or structural differences which exist when these proteins fold separately rather than as a 2B-3_{181} polyprotein. The failure of YF NS2B proteins containing deletions in the conserved domain to associate with the NS3 proteinase domain in coimmunoprecipitation experiments is consistent with the hypothesis that either this domain forms a binding site for the proteinase domain or the domain directly affects a site on NS2B which is important for interaction with NS3. Although a more complete understanding of complex formation could be obtained by testing complex formation in *trans* with vaccinia virus NS2B recombinants containing mutations in the hydrophobic regions, analysis of in vitro-translated 2B-3_{181} polyproteins containing these mutations revealed that, under nondenaturing conditions, coimmunoprecipitation of NS2B and 3_{181} occurs (data not shown). The clustering of charged residues in the conserved domain is consistent with its function as a site for interaction between proteins (17). The predicted cytoplasmic orientation of this region, based on its hydrophilic character and the topology of the surrounding hydrophobic regions (30), is consistent with the hypothesis that NS2B may be a membrane-associated protein which interacts with the cytoplasmic NS3 and localizes it to membranes. Although it has been reported that only a fraction of NS3 is localized to membranes in the presence of NS2B (13), it is possible that more than one protein, possibly even host proteins, may participate in association of NS3 with membranes. Localization of NS3 to the membrane may be necessary for processing of the viral polyprotein or for function of the replication complex, both of which are believed to be membrane-associated processes (see reference 7 for a review). On the basis of the current data, we favor a model in which association of the proteinase domain with the NS2B conserved domain occurs prior to and is necessary for 2B/3 cleavage and is followed by formation of a stable complex. A conformational change may occur in conjunction with 2B/3 cleavage which enhances proteinase activity, since 2B-3_{181} polyproteins which are blocked for cleavage at the 2B/3 site have reduced cleavage activity in *trans* relative to a *cis*-processed wt 2B-3_{181} proteinase (10). A possible role for NS2B may be to stabilize NS3 in an active conformation. Factors which disrupt this interaction, by association with either NS2B or NS3, could be important for regulating proteinase activity during virus infection. However, we cannot

exclude the possibility that the conserved domain functions to stabilize the structure of NS2B and that the site for interaction with the NS3 proteinase domain does not directly involve this region. More comprehensive genetic and biochemical and ultimately high-resolution structural studies will be required to precisely define the site(s) of interaction.

In contrast to the variable effects that mutations in NS2B had on polyprotein processing, all mutations tested had lethal effects on viral replication as tested by the recovery of infectious plaque-forming virus. The deleterious effects of mutations which abolish or significantly reduce proteolytic processing (CD3 and CD4) are similar to results previously obtained with mutations at Ser-138 of the NS3 proteinase domain. Although processing appeared normal with the 1D3 and 2D4 mutations, and only slightly altered with the 3D3 mutation, these were all lethal for replication. The precise stage at which the mutations block replication has not been defined. It is possible that these mutations disrupt either the assembly or the function of the replication complex, which has been proposed to consist of the NS3 and NS5 proteins together with the proteins derived from the NS2 and NS4 subregions (reviewed in reference 7). The precise interactions between NS2B and these additional viral proteins remain to be defined, although coimmunoprecipitation experiments with YF-infected cell extracts with antisera to NS4B and NS5 suggest that stable interactions may occur (10). It has been observed for poliovirus that some mutations in the hydrophobic domain of the 3A region interfere with RNA synthesis in some cases without abolishing polyprotein processing (16). In addition, mutations within the proposed FG loop of the poliovirus 3C proteinase also exhibit disparate effects on proteolytic processing and viral replication (19). It would appear from examples such as these that polyprotein processing and assembly of processed proteins into functional replication complexes are discrete steps, and proteins which are involved in processing and replication may have several functions. Whether the NS2B mutations studied here act solely through altering the association of NS2B and NS3, or by interfering with other functions of NS2B, remains to be determined.

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