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Mutagenesis of the Yellow Fever Virus NS2B/3 Cleavage Site: Determinants of Cleavage Site Specificity and Effects on Polyprotein Processing and Viral Replication

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The determinants of cleavage site specificity of the yellow fever virus (YF) NS3 proteinase for its 2B/3 cleavage site have been studied by using site-directed mutagenesis. Mutations at residues within the GARR ↓ S sequence were tested for effects on *cis* cleavage of an NS2B-3$_{181}$ polyprotein during cell-free translation. At the P1 position, only the conservative substitution R→K exhibited significant levels of cleavage. Conservative and nonconservative substitutions were tolerated at the P1′ and P2 positions, resulting in intermediate levels of cleavage. Substitutions at the P3 and P4 positions had no effects on cleavage efficiency in the cell-free assay. Processing at other dibasic sites was studied by using transient expression of a sig2A-5$_{356}$ polyprotein. Cleavage at the 2B/3 site was not required for processing at downstream sites. However, increased accumulation of high-molecular-weight viral polyproteins was generally observed for mutations which reduced cleavage efficiency at the 2B/3 site. Several mutations were also tested for their effects on viral replication. Virus was not recovered from substitutions which blocked or substantially reduced cleavage in the cell-free assay, suggesting that efficient cleavage at the 2B/3 site is required for flavivirus replication.

Yellow fever virus (YF), like all members of the family *Flaviviridae*, contains a single positive-strand RNA genome encoding a polyprotein precursor which is processed into at least 10 viral proteins (3, 19). Previous studies have established that cleavages generating the N termini of the nonstructural proteins NS2B, NS3, NS4A, and NS5 occur following two basic amino acid residues within a consensus sequence defined as G(A)RR ↓ S(G) (reviewed in references 3 and 20). Inspection of a number of flavivirus cleavage site sequences indicates that the P1′, P1, and P2 positions are highly conserved for short side chain (P1′) and basic amino acid residues (P1, P2), respectively (3). Evidence for alternative cleavages within the NS2A and NS4A regions defined by the sequences QK ↓ T and QR ↓ S, respectively, has also been obtained (13, 15). All of these cleavages are mediated by a serine proteinase domain contained within the N terminus of the YF NS3 protein (2, 8). Homology studies and site-directed mutagenesis of the YF proteinase implicate a catalytic triad (His-53, Asp-77, Ser-138) and a substrate binding pocket (located at residues 151 to 156) in cleavage activity of the proteinase (1, 8, 10, 16). In addition, the small hydrophobic nonstructural protein NS2B has been shown to be essential for proteinase activity, and a conserved domain within NS2B is required for NS2B-NS3 proteinase complex formation and function (6). Previous work has also suggested that cleavage at the 2B/3 and 2A/2B cleavage sites occurs in *cis*, whereas cleavage at the 4B/5 site may occur efficiently in *trans* (2, 8), suggesting that different substrate requirements may exist for cleavage at certain sites. Although studies with chimeric proteinases between YF and dengue virus type 2 have implicated residues in the proposed substrate binding pocket as important for cleavage site specificity (16), the relative importance of amino acid residues within the consensus cleavage site as determinants of cleavage efficiency has not been thoroughly examined. Mutagenesis of the YF 4B/5 and 3/4A cleavage sites reveals that the P1′ exhibits a stringent requirement for residues with a small side chain, whereas at the P1 and P2 positions, basic residues are preferred but not essential for cleavage (14). Similar findings have been obtained for the P1′, P1, and P2 positions of the YF 2A/2B cleavage site; however, the P3 and P4 positions may also influence cleavage efficiency at this site (15). In order to further characterize the importance of residues within the consensus sequence for cleavage efficiency, mutagenesis of the P4 through P1′ positions of the YF 2B/3 cleavage site was carried out and analyzed in cell-free and cellular expression systems. To understand the importance of cleavage efficiency at this site for replication of the virus, several mutations were analyzed for their effects on recovery of infectious YF after transfection with full-length RNA transcripts harboring these mutations.

MATERIALS AND METHODS

Cells and viruses. Growth of SW-13 cells and infection with YF17D strain has been described previously (4). Preparation of vTF7-3, the vaccinia virus expressing T7 RNA polymerase (9), has been described elsewhere (2).

T7 transcription plasmids. The use of pET-8c plasmids (22) for cloning and expression of YF proteins under control of the T7 promoter has been previously described (2, 8). Plasmid constructions were generated by using standard methods and reagents (21), and the structures were verified by restriction enzyme digests and nucleotide sequence analysis.

Plasmid constructions. Construction of pET-8c-NS2B-3$_{181}$, a plasmid encoding the YF NS2B protein and the NS3 proteinase domain, and pET/BS(+)NS2B-3$_{181}$, a corresponding phagemid allowing rescue of single-stranded DNA containing the YF plus strand, has been described previously (8). Mutations at the 2B/3 cleavage site were made by oligonucleotide-directed mutagenesis (11) using uridylated phagemid DNA as the template and oligonucleotide primers degenerate for codons specifying each of the P1′, P1, P2, P3, and P4 residues of the 2B/3 cleavage site.

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In vitro transcription. 5′ capped transcripts were produced from linearized pET-8c-NS2B-3_{181} plasmid DNA templates, using T7 RNA polymerase and conditions recommended by the manufacturer (Epicenter).

Cell-free translation. Cell-free translation was performed by using rabbit reticulocyte lysates programmed with pET8c-2B-3_{181} RNA transcripts in accordance with the manufacturer's specifications (Promega). Proteins were labelled by inclusion of [^{35}S]methionine (Amersham).

Transient expression assay. Expression of YF polyproteins by using the transient expression assay has been described (2). Briefly, confluent monolayers of SW-13 cells were infected with vTF7-3, followed by DNA transfection using 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.

Immunoprecipitation. Extraction of YF-infected SW-13 cells for YF-specific proteins was as previously described (4). Lysates from transient expression experiments were prepared by using denaturing conditions, and immunoprecipitation was performed as described previously (4). Immunoprecipitates were collected by using *Staphylococcus aureus* Cowan strain I (Calbiochem) and solubilized in Laemmli sample buffer.

Gel electrophoresis. Washed immunoprecipitates were solubilized in sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (12), using methods previously described (4).

Construction of full-length YF cDNA templates and RNA transcription and transfection. pET-8c-sig2A-5_{356} plasmids containing 2B/3 cleavage site mutations were used to reconstruct full-length YF cDNA clones by using methods previously described (17), with slight modifications. The *Avr*II-*Nhe*I fragment from pET-8c-sig2A-5_{356} clones containing the relevant mutation was subcloned into the YFM5.2 plasmid, and the *Aat*II-*Nsi*I fragments of the YFM 5.2 clones and YF5′3′IV were ligated after recovery of appropriate fragments from low-melting-point temperature agarose gels. By using this method, approximately 100 ng of full-length template was generated, as judged from analysis by agarose gel electrophoresis. Full-length templates were linearized with *Xho*I, and SP6 transcripts were synthesized as previously described (17). Yield and integrity of YF transcripts was determined by nondenaturing RNA gel electrophoresis. Transfection of SW-13 cells was carried out by using conditions described for BHK-21 cells (17). Briefly, cell monolayers were washed with phosphate-buffered saline (PBS) and transfected with a mixture of PBS, Lipofectin reagent, and RNA transcripts for 10 min at room temperature. The transfection mixture was then removed, cells were washed with minimal essential medium (MEM), and then the monolayer was overlaid with 1% agarose in MEM containing 2% fetal bovine serum and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). 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–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 plaque assays were performed. Plaque assays were performed on SW-13 cells by using overlay conditions described above.

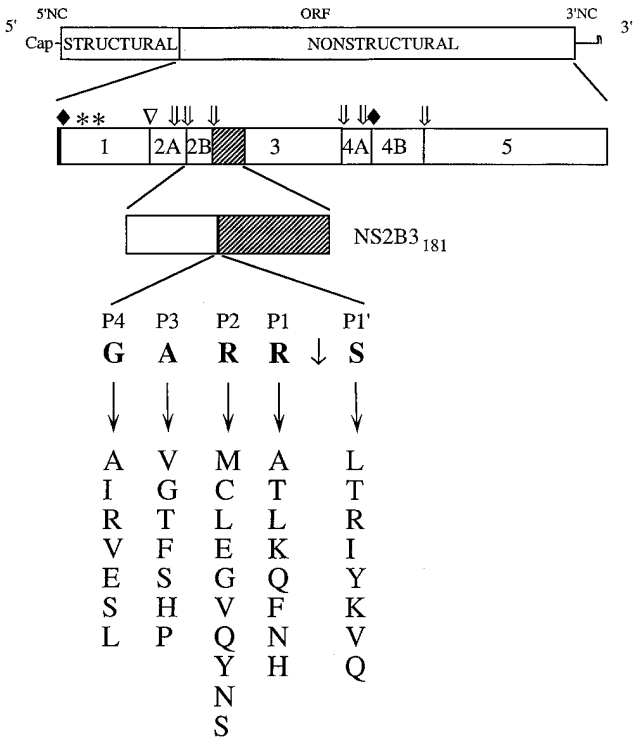

FIG. 1. Site-directed mutagenesis of the YF 2B/3 cleavage site. The YF genome with its structural and nonstructural regions is depicted at the top. Below is indicated the structure of the sig2A-5_{356} polyprotein and the individual amino acid substitutions engineered at the P1′, P1, P2, P3, and P4 positions of the 2B/3 cleavage site. The single-letter code for amino acids is used. The construction of pET-8c sig2A-5_{356} and detailed analysis of the expression and proteolytic processing of the encoded polyprotein have been described in detail elsewhere (2). The NS3 proteinase domain is shaded, and cleavages mediated by the NS2B-NS3 proteinase are indicated by open arrows. Solid diamonds indicate signalase cleavages at the N terminus of sig2A-5_{356} and at the 2K/4B site (13). Asterisks indicate N-linked glycosylation sites within NS1. NC, noncoding region; ORF, open reading frame.

RESULTS

Generation of mutants. Expression of the NS2B-3_{181} polyprotein was used to assay the effects of amino acid substitutions on cleavage efficiency at the 2B/3 cleavage site. Previous work has shown that during expression in reticulocyte lysate, this polyprotein undergoes site-specific cleavage at the 2B/3 cleavage site and that this cleavage is dependent on the presence of an intact serine proteinase catalytic triad within the NS3 proteinase domain (8). This cleavage event has been shown to be dilution insensitive during cell-free translation and is therefore believed to reflect a *cis* processing event which presumably also occurs during processing of the viral polyprotein in infected cells (5, 8). Figure 1 illustrates the individual mutations introduced at the P1′ through P4 positions of the 2B/3 cleavage site. The effects of single amino acid changes at these positions on cleavage at the 2B/3 site were analyzed by immunoprecipitation of NS2B-3_{181} polyproteins and their cleavage products generated during cell-free translation. Immunoprecipitation was necessary to facilitate identification of the 14-kDa NS2B protein, which is otherwise obscured because of comigration with hemoglobin during SDS-polyacrylamide gel electrophoresis.

Effects of P1′ mutations on 2B/3 cleavage. Figure 2A illustrates the cleavage activity of NS2B-3_{181} polyproteins containing substitutions at the P1′ position. None of the mutations introduced at this position abolished cleavage. Compared with the wild-type polyprotein (lane S), which undergoes almost complete cleavage during 1 h of cell-free translation, several mutations allow substantial levels of cleavage, as judged by the relative amount of NS2B generated. These include the conservative substitution T, but also the uncharged polar substitutions Y and Q. Among the hydrophobic substitutions, L and I were not well tolerated, whereas the smaller V residue allowed cleavage. Surprisingly, the nonconservative substitutions R and K did not abolish cleavage. NS2B proteins derived from polyproteins containing R or K at P1′ migrated with apparent molecular weights slightly greater than that of wild-type NS2B. Substitution of a basic residue (R or K) at P1′ creates a potential consensus cleavage site defined by ARRR/K ↓ G, with a conserved G at P1′. Since substitutions at P3 and P4 do not affect cleavage efficiency (see below), this suggests that the proteinase cleaves at this alternative site and that the structural context provided by adjacent polyprotein sequences is flexible enough to permit this cleavage. The 3_{181} cleavage products generated from mutations at P1′ exhibited different apparent molecular weights, and the relative amounts were variable. These differences are presumably due to modification of the N terminus of the 3_{181} cleavage product, with effects on both electrophoretic migration and stability in the cell-free system. Higher-molecular-weight proteins were also immunoprecipitated from the translation reactions. These proteins have not been further characterized.

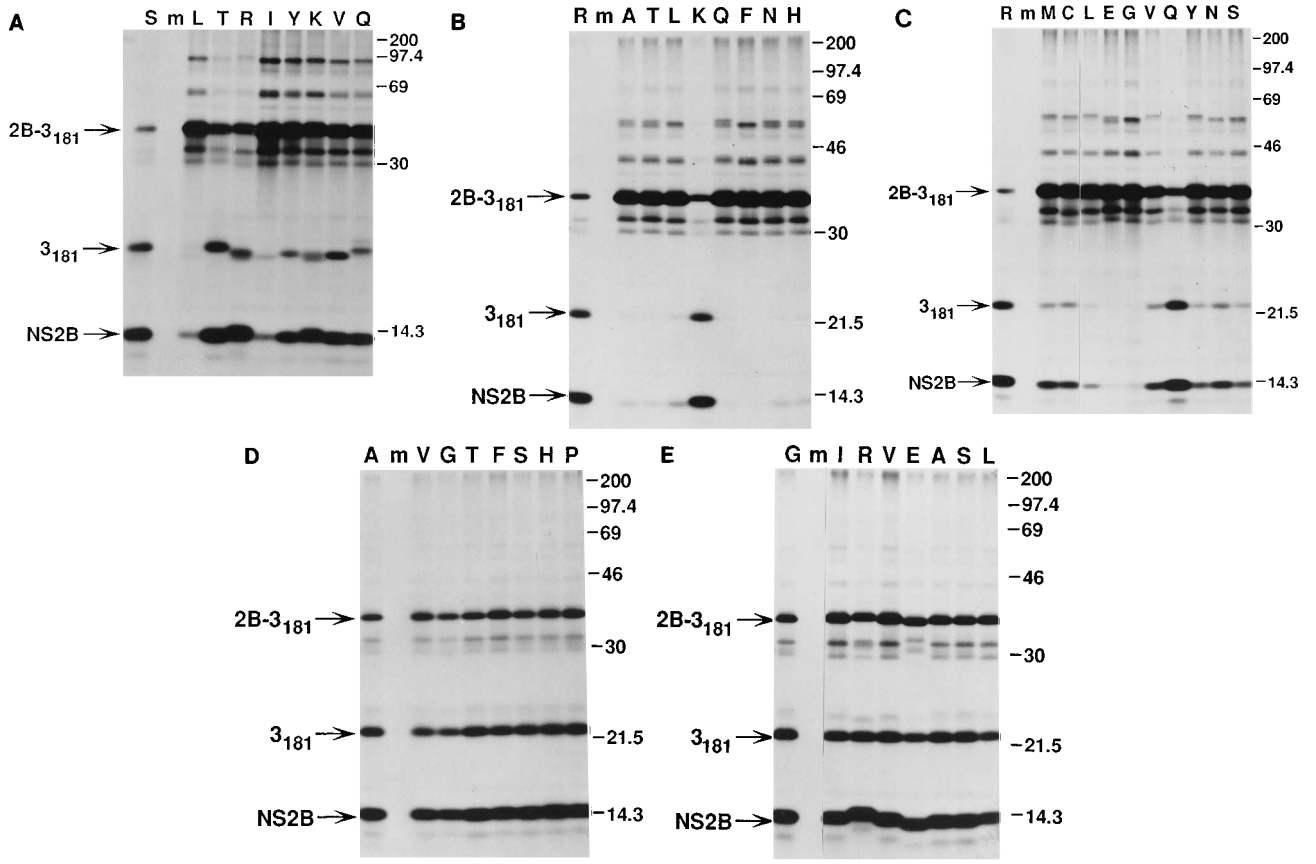

FIG. 2. Effects of mutations at the 2B/3 cleavage site on NS2B-3_{181} processing. Rabbit reticulocyte lysates were programmed with RNA encoding parental or mutant NS2B-3_{181} polyproteins as described in Materials and Methods. Following 1 h of translation, viral proteins were immunoprecipitated with a mixture of antisera specific for the YF NS2B and NS3 proteins, using conditions previously described (8). Immunoprecipitates were run on SDS–15% polyacrylamide gels, and ^{35}S-labeled proteins were visualized by fluorography. Mutations are indicated at the top of the gel, using the single-letter amino acid code. The parental residue is indicated at the far left of each panel. Lane m indicates mock-programmed lysates (no RNA). Positions of the 2B-3_{181} polyprotein and the 3_{181} and NS2B cleavage products are indicated to the left. Positions and sizes (in kilodaltons) of ^{14}C-labeled molecular weight standards are indicated on the right. (A) P1′ mutations; (B) P1 mutations; (C) P2 mutations; (D) P3 mutations; (E) P4 mutations.

Effects of P1 mutations on 2B/3 cleavage. Figure 2B illustrates the cleavage activity of NS2B-3_{181} polyproteins containing substitutions at the P1 position. With the exception of the conservative substitution of K for R, all mutations including residues with charged, polar, hydrophobic, or short side chains led to drastically reduced levels of cleavage. Replacement with F abolished cleavage within limits of detection of this system. An R residue at the P1 position is highly conserved at the consensus cleavage sites within the YF nonstructural polyprotein and also the consensus cleavage site of other flaviviruses. These data suggest that the P1 position is an important determinant of cleavage efficiency at the 2B/3 cleavage site.

Effects of P2 mutations on 2B/3 cleavage. Figure 2C illustrates the cleavage activity of NS2B-3_{181} polyproteins containing substitutions at the P2 position. No substitutions abolished cleavage. Replacement with the polar Q at the P2 but not P1 position led to levels of cleavage similar to wild-type levels. It is notable that the 2B/3 cleavage sites of three serotypes of Dengue virus contain Q at P2 (3). Substitution of residues with other uncharged polar (Y, N, S, C) or nonpolar (M, L) R groups was in general tolerated, although G drastically reduced cleavage. Introduction of a negatively charged residue (E) at P2 also markedly reduced cleavage.

Effects of P3 mutations on 2B/3 cleavage. The P3 position is not highly conserved among YF consensus cleavage sites or among flavivirus 2B/3 cleavage sites, although S, T, G, or A residues are often found. This is reflected in the lack of an effect of substitutions at this position on cleavage efficiency of the YF NS2B-3_{181} polyprotein (Fig. 2D). Introduction of the residues often found at the P3 position of cleavage sites of other flaviviruses (S, T, or G) had no significant effect on cleavage efficiency. A range of nonconservative mutations (H, P, F, V) introduced at P3 also resulted in levels of cleavage similar to wild-type levels, although minor differences were apparent.

Effects of P4 mutations on 2B/3 cleavage. Figure 2E illustrates the effects of substitutions at P4 on the cleavage efficiency of the NS2B-3_{181} polyprotein. P4, like P3, is not a conserved position within consensus cleavage sites of YF or other flaviviruses. A range of conservative and nonconservative mutations had little effect on cleavage efficiency relative to the wild-type residue. In particular, introduction of a charged residue did not appear to impair cleavage. For the R and E substitutions, differences were observed in the apparent molecular weight of the NS2B cleavage product. This is not consistent with an aberrant cleavage event since the apparent size of the 3_{181} cleavage product was unaltered (Fig. 2A) and may result from the effect of a positive or negative charge on the electrophoretic migration of the NS2B cleavage product.

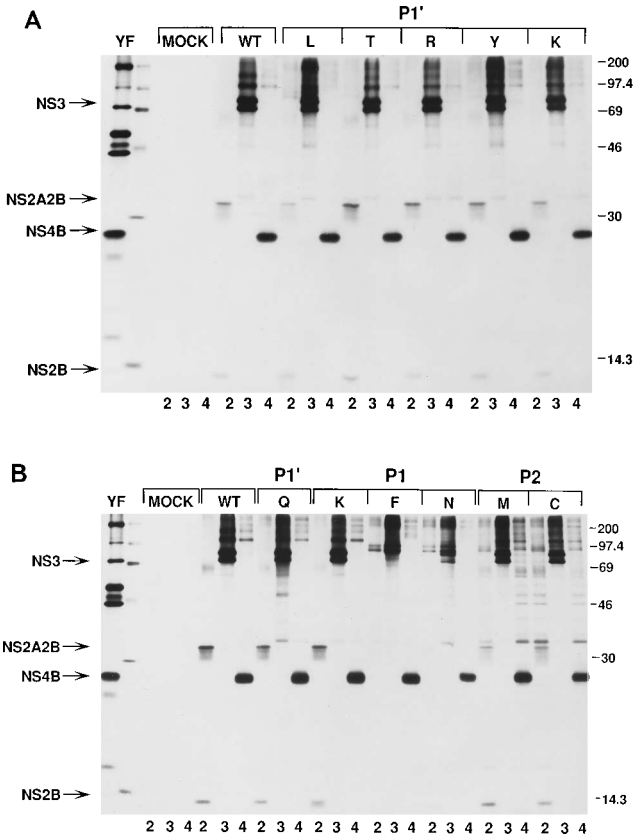

FIG. 3. Effects of 2B/3 cleavage site mutations on processing of the sig2A-5_{356} polyprotein, as examined by transient expression in the vaccinia virus-T7 system (2). Infection of SW-13 cells with vTF7-3 and transfection with pET-8c-sig2A-5_{356} plasmid DNA was as described in Materials and Methods. Cells were labelled for 4.5 h with [^{35}S]methionine, SDS lysates were prepared, and immunoprecipitation was carried out as described in Materials and Methods. Numbers at the bottom refer to the antisera specific for the NS2B, NS3, and NS4B proteins, which were analyzed separately for each construct. Mock indicates cells infected with vTF7-3 but not transfected with DNA. WT indicates the parental construct. The positions of the viral nonstructural proteins produced by processing of this polyprotein are indicated to the left. Positions (in kilodaltons) of the molecular weight markers (present in lane 2) are shown to the right. YF indicates YF proteins immunoprecipitated with mouse hyperimmune ascitic fluid (4). Proteins were run on an SDS–13% polyacrylamide gel and then subjected to fluorography. (A) Mutations at P1′; (B) mutations at P1′, P1, and P2.

Analysis of nonstructural protein processing, using transient expression of a sig2A-5_{356} polyprotein. In order to characterize the possible effects of these mutations on processing elsewhere in the nonstructural region, selected mutations were introduced into a sig2A-5_{356} polyprotein for transient expression analysis after DNA transfection (2). As previously demonstrated, this polyprotein undergoes processing at consensus cleavage sites in the presence of an intact NS3 proteinase domain, and this system is believed to mimic authentic processing (2). Viral proteins and polyproteins were identified by immunoprecipitation with NS2B-, NS3-, or NS4B-specific antisera. Compared with the wild-type polyprotein, all mutant constructs except P1 F and P1 N generated readily detectable levels of the NS2-related proteins, which include NS2B (14 kDa) and the 30- to 33-kDa NS2A-2B cleavage products (Fig. 3). The 27-kDa NS4B protein was produced by the wild-type and all mutant polyprotein constructs. Noticeable differences were observed in the processing of NS3-specific proteins. Wild-type NS3-related proteins include 70- and 75-kDa species and at least two discrete higher-molecular-weight species which also immunoprecipitate with antiserum to NS4B. Polyproteins containing the T or R substitution at the P1′ position or K at the P1 position, all of which permitted recovery of infectious virus (see below), generated a profile of NS3-related proteins similar to the wild-type pattern. Polyproteins containing mutations which abolished recovery of virus (P1′ L, Y, K, or Q; P1 F or N; P2 M or C) usually generated altered processing patterns. As expected from the results of the cell-free translation studies, polyproteins containing F or N at P1 were defective for cleavage at the 2B/3 site, yielding markedly reduced levels of mature 70-kDa NS3 and increased levels of NS2- and NS3-specific polyproteins. In addition, these and other mutations affecting cleavage at the 2B/3 site exhibited a higher proportion of even larger polyproteins. This may indicate that reduced processing at the 2B/3 site leads to less efficient processing at other sites in the NS3-4-5 region.

TABLE 1. Effects of 2B/3 cleavage site mutations on viral replication[a]

Expt	Mutation	Cleavage[b]	PFU[c]	Titer[d]	Plaque size[e]
1	None (WT)	+++	110	6.5	WT
	P1′ L	+	0	<0	
	P1′ T	++	179	5.0	Small
	P1′ R	++	104	4.5	Small
	P1′ Y	++	0	<0	
	P1′ K	++	0	<0	
	P1′ Q	++	0	<0	
2	None (WT)	+++	109	7.6	WT
	P1 K	+++	102	6.5	Small
	P1 F	−	0	<0	
	P1 N	+/−	0	<0	
	P2 M	+	0	<0	
	P2 C	+	0	<0	
	P3 G	+++	>200	7.6	WT
	P3 F	+++	90	7.8	Small
	P3 H	+++	>200	7.4	WT
	P4 I	+++	96	6.2	Small
	P4 R	+++	>200	8.3	WT
	P4 E	+++	>200	6.7	Small

[a] All transfections were repeated at least once. SW-13 cells were transfected with RNA transcripts as described in the text. WT, wild type.
[b] The estimated efficiency of cleavage at 2B/3 based on the cell-free cleavage assay.
[c] Plaques obtained following transfection with 250 nanograms of RNA.
[d] Log_{10} of the viral titer of the media harvested from the monolayers at 60 h following transfection.
[e] Relative plaque size at 96 h after transfection.

Effects of 2B/3 cleavage site mutations on recovery of infectious virus. Previous studies with mutations at the YF 2A/2B cleavage site indicated that in general, mutations which abolished cleavage were lethal for recovery of infectious virus, whereas mutations which only reduced cleavage had variable effects on recovery (15). In order to determine the biological significance of altered cleavage efficiency at the 2B/3 cleavage site, several mutations were introduced into full-length YF cDNA templates and the infectivity of transcripts for SW-13 cells and properties of recovered virus were determined. Table 1 illustrates the results of these experiments. At the P1′ position, substitution of S with L, Y, K, V, or Q resulted in failure to recover infectious virus. Substitution of T or R yielded infectious virus, and specific infectivities were similar to those for wild-type transcripts. Titers of virus recovered from the media of the transfections at 60 h differed considerably from that of wild-type virus, which yielded 3×10^6 PFU/ml. The P1′ T mutation yielded 10^5 PFU/ml, and the plaque size of recovered virus was smaller than that of the wild-type virus. The P1′

R mutation yielded less virus (3×10^4 PFU/ml) than wild type and exhibited a small-plaque phenotype. At the P1 position, only the conservative mutation P1 K generated infectious virus. Titers were less than wild-type titers (3×10^6 versus 4.5×10^7 PFU/ml), and the plaque size was smaller. Transcripts encoding substitution of F or N for R at P1 were noninfectious. Similar results were obtained for two mutations at the P2 position, where substitution of M or C for R resulted in failure to recover infectious virus. At the P3 and P4 positions, all transcripts tested were infectious, although specific infectivities varied and some of the recovered viruses exhibited growth properties and plaque phenotypes distinct from those of the wild-type parent (Table 1).

These results indicate that 2B/3 cleavage site mutations which dramatically reduce or abolish cleavage (P1′ L, Y, K, Q; P1 F, N; P2 M, C) eliminate productive replication in SW-13 cells. Only mutations with less severe or no observable effects on cleavage efficiency in the cell-free assay allowed recovery of virus.

DISCUSSION

In previous studies involving mutagenesis of the YF 2A/2B, 3/4A, and 4B/5 nonstructural cleavage sites, a range of effects on cleavage efficiency were observed and evidence for distinct effects of substitutions at individual sites was obtained (14, 15). Mutations which impaired cleavage at 2A/2B were also found to be deleterious for viral replication. Because these studies analyzed only limited mutations, the current investigation was designed to gain a more complete understanding of the specificity of the YF NS3 proteinase for its consensus cleavage site and, additionally, to further investigate cleavage site mutations for their effects on viral replication. In contrast to the previous studies, a cell-free translation assay was utilized to characterize the 2B/3 cleavage site mutations, since 2B-3_{181} polyproteins undergo nearly quantitative, site-specific cleavage in this system (8). It is possible that certain factors may influence proteinase activity and therefore cleavage efficiency when cellular and cell-free systems are compared; however, we believe that the cell-free system is a more reproducible assay for analyzing NS3 proteinase activity, in lieu of having a purified proteinase preparation. We chose to analyze effects of mutations at the P1′ through P4 positions, since these are the most highly conserved positions among flavivirus nonstructural cleavage sites (3), although we cannot disregard the effects of residues outside of this core sequence as potentially important for cleavage efficiency.

Results of the cell-free translation assay indicate that for the 2B/3 cleavage site, substitutions at the individual positions of the GARR ↓ S cleavage site have markedly different effects on cleavage efficiency. The P1 position exhibits a stringent requirement for a basic amino acid, with all other substitutions essentially eliminating cleavage. A similar result has been obtained for the 2A/2B cleavage site (15); however, the P1 position of the 4B/5 site is tolerant of nonconservative substitutions (14). The high stringency for a basic residue at P1 is also reflected in the results of substitutions at P1′, where the profile of cleavage products suggests that aberrant cleavage with R or K retained at P1 is favored over substitution of a basic residue at this position. For 2B/3, the P1′ position is otherwise tolerant of a range of substitutions. This is in contrast to the 4B/5 and 2A/2B cleavage sites, where preference for a short side chain amino acid has been demonstrated (14, 15). The P2 position of the 2B/3 cleavage site also is tolerant of a range of substitutions, whereas nonconservative changes at the 2A/2B and 4B/5 cleavage sites lead to reduced cleavage efficiency. It is notable that no significant effects on cleavage efficiency by mutations introduced at the P3 and P4 positions of the 2B/3 cleavage site were observed. Some substitutions at these positions of the 2A/2B site reduced cleavage efficiency, whereas others appeared to enhance cleavage, suggesting that the wild-type residues were not optimal for cleavage (15). The current data, taken together with these previous studies, support several conclusions regarding cleavage site specificity of the NS3 proteinase. There is a general requirement for a basic residue at P1, as was originally suggested (18), particularly for cleavage at the 2A/2B and the 2B/3 sites. Residues with small aliphatic side chains are preferred at P1′, particularly for the 2A/2B and 4B/5 cleavage sites. The presence of a basic residue at P2 also appears to be favored, especially at the 2A/2B site. It is interesting that whereas all YF proteinase cleavage sites contain R at the P2 position, the dengue virus serotypes have evolved with Q at this position, suggesting that the structural constraints of the proteinase, particularly in the interaction of P2 with the substrate binding pocket, may have more flexibility than at the P1 position. This may reflect a higher priority for an acceptable geometry provided by certain P2 substitutions rather than a charge interaction. Available evidence also supports the hypothesis that P3 and P4 residues can influence cleavage efficiency, as judged from results obtained at the 2A/2B cleavage site (15). The data reported here suggest that for the 2B/3 site, the P3 and P4 residues have evolved to confer optimal cleavage efficiency, which may allow preferential cleavage at the 2B/3 site and establish a preferred order for polyprotein processing.

We have examined processing of sig2A-5_{356} polyproteins to address whether mutations at the 2B/3 site would alter cleavage efficiency at other NS3 proteinase-dependent cleavage sites in the nonstructural region. Studies with mutations at the 3/4A and 4B/5 cleavage sites have suggested that there is no obligate order for processing of the nonstructural polyprotein (14). This also appears to be the case for substitutions blocking cleavage at the 2B/3 site since NS4B was efficiently produced by the P1 F mutant. However, we did observe that mutations which reduced the efficiency of cleavage at the 2B/3 site in the cell-free assay (most P1′, P1, and P2 substitutions) were usually associated with altered profiles of high-molecular-weight polyproteins in the transient expression assay. It is possible that altering the cleavage efficiency at this site may perturb the interaction of NS2B with the NS3 proteinase domain during formation of the proteinase complex from the nascent NS2B-3 polyprotein. This in turn may slow the rate of cleavage at other sites (3/4A, 4A/4B, 4B/5) or lead to aberrant cleavage, perhaps because folding events render these sites in a less optimal conformation for processing when 2B/3 cleavage is delayed.

We have extended the analysis of cleavage site mutations to determine the significance of alterations in cleavage efficiency on viral replication, by studying selected mutations in the context of a replication cycle initiated by transfection with RNA transcripts. The results indicate that mutations which minimally reduce cleavage efficiency (such as the R or T substitution at the P1′) may still allow viral replication; however, mutations which cause more severe reductions in cleavage efficiency and are associated with altered polyprotein processing in transient expression experiments do not permit recovery of virus. It is also apparent that mutations which allow efficient processing at the 2B/3 cleavage site (P3 and P4 substitutions) can still lead to altered growth properties such as reduced or enhanced virus yield or small plaque size. This phenomenon has also been observed with mutations at the 2A/2B cleavage site (15). It is possible that alterations in the structure of the NS2B and NS3 proteins caused by the cleavage site mutations

influence the properties of these proteins as components of the viral replication machinery. Even subtle differences in cleavage efficiency not detectable in our processing assays may influence the protein interactions which may be required for assembly of functional replication complexes. The precise blocks caused by mutations which abolish recovery of infectious virus have not been determined; however, we have observed that in such cases, viral polyproteins are not observed during labelling experiments performed 48 h after transfection (7). This suggests that these mutations create an early block to viral replication, perhaps at the level of initiation of RNA synthesis. We are attempting to confirm these findings by analyzing the RNA phenotypes of cleavage site mutants which are either blocked for replication or associated with reduction in virus yield. This is likely to advance our understanding of the relationships of processing events to the initiation of the replication cycle as well as elucidate functions of polyproteins and cleavage products in RNA replication and possibly other processes involved in the viral life cycle.

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