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cis-Acting Lesions Targeted to the Hydrophobic Domain of a Poliovirus Membrane Protein Involved in RNA Replication

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The structural requirements of the hydrophobic domain contained in poliovirus polypeptide 3AB were studied by using a molecular genetic approach in combination with an in vitro biochemical analysis. We report here the generation and analysis of deletion, insertion, and amino acid replacement mutations aimed at decreasing the hydrophobic character of the domain. Our results indicated that the hydrophobicity of this region of 3AB is necessary to maintain normal viral RNA synthesis. However, in vitro membrane association assays of the mutated proteins did not establish a direct correlation between 3AB membrane association and viral RNA synthesis. Some of the lethal mutations we engineered produced polyproteins with abnormal P2- and P3-processing capabilities due to an alteration in the normal cleavage order of the polyprotein. A detailed analysis of these mutants suggests that P2 is not the major precursor for polypeptides 2A and 2BC and that P2 protein products are derived from P2-P3-containing precursors (most likely P2-P3 or P2-3AB). Such precursors are likely to result from primary polyprotein cleavage events that initiate a proteolytic cascade not previously documented. Our results also indicated that the function provided by the hydrophobic domain of 3AB cannot be provided in *trans*. We discuss the implications of these results on the formation of limited-diffusion replication complexes as a means of sequestering P2- and P3-region polypeptides required for RNA synthesis and protein processing.

Poliovirus (PV), a member of the *Picornaviridae* family, has a single-stranded RNA genome of approximately 7,500 nucleotides. A small viral protein, VPg, is covalently attached to its 5′ end, and a genetically encoded poly(A) tail is attached at its 3′ end. The viral genome acts as mRNA to direct the synthesis of a single polypeptide, which is efficiently processed by three viral proteases (3C, 2A, and 3CD) into several viral polypeptides (Fig. 1). While the proteins encoded by the P1 region of the genome, VP1, VP2, VP3, and VP4, are capsid proteins, the P2 and P3 regions of the genome encode nonstructural proteins, most of them required for viral RNA (vRNA) synthesis (for reviews, see references 30, 49, and 54).

Although the cycle of PV replication was described more than 20 years ago, the molecular details of RNA synthesis are still not well understood. For replication, the genomic (plus-strand) RNA is used as a template to synthesize a complementary (minus) RNA strand which in turn is used as template for plus-strand synthesis. In vivo RNA synthesis takes place in replication complexes associated with the outer surfaces of virus-induced vesicles present in the rough endoplasmic reticulum (6, 11). Replication complexes are formed by vRNA in a partially double-stranded replicative intermediate structure in association with RNA replication proteins (9, 10, 20, 38, 48). A combination of genetic and biochemical studies have begun to define the specific functions performed by some of the viral proteins. These studies have also provided evidence for the involvement of cellular proteins in PV RNA synthesis. For example, electron microscopy and biochemical studies suggested that P2-derived proteins (2C and probably its precursor 2BC) attach the vRNA to the vesicular membranes, providing the correct spatial organization of the RNA necessary for its replication (7). Analysis of several mutants in 2B and 2C confirmed their involvement in RNA synthesis (5, 36, 46) and, in particular, for 2B (or its precursor 2BC), in vRNA amplification (25).

Among the proteins derived from the P3 region, polypeptide 3D is the primer- and RNA-dependent RNA polymerase. It is the only protein required for elongation of RNA chains in vitro (15, 16, 37, 61). Although elongation is thought to be the primary function of 3D, there is evidence that the polymerase is involved in other functions related to RNA synthesis. Genetic studies indicated that 3D is able to uridylylate VPg, its proposed primer (60). There is also evidence that 3D, in association with 3C and a cellular protein(s), forms a ribonucleoprotein complex with the first 100 nucleotides of the genome. The exact function of this complex is not known, but interestingly, it is only required for plus-strand RNA synthesis (2). In addition, the P3-derived proteins VPg (3B), 3A, and their precursor, 3AB, are involved in RNA synthesis. Bernstein and Baltimore (4) reported the isolation of a cold-sensitive PV mutant containing a 3A lesion that is defective in RNA synthesis, and a study of several mutations engineered in VPg indicated that the covalent attachment of VPg to viral RNA is an absolute requirement for RNA synthesis (47).

It has been proposed that VPg, a polypeptide precursor of VPg, or a uridylylated derivative of VPg is the primer for the vRNA polymerase (40, 42, 56, 57). This proposal is supported by the following observations. (i) VPg is linked to the 5′ end of both the plus and minus strands of RNA, as well as to all the nascent RNAs (42, 45). (ii) Antibodies against VPg immunoprecipitate VPg as well as VPgpU(pU) from infected cells (13). (iii) In in vitro replication reactions, anti-VPg antibody specifically inhibits initiation of vRNA synthesis (3, 39). (iv) In vitro synthesis of VPgpU and subsequent chase of this protein-nucleotidyl moiety to VPgpUpU and nucleotidyl proteins containing nine or more of the PV 5′-proximal nucleotides were demonstrated in a membranous replication

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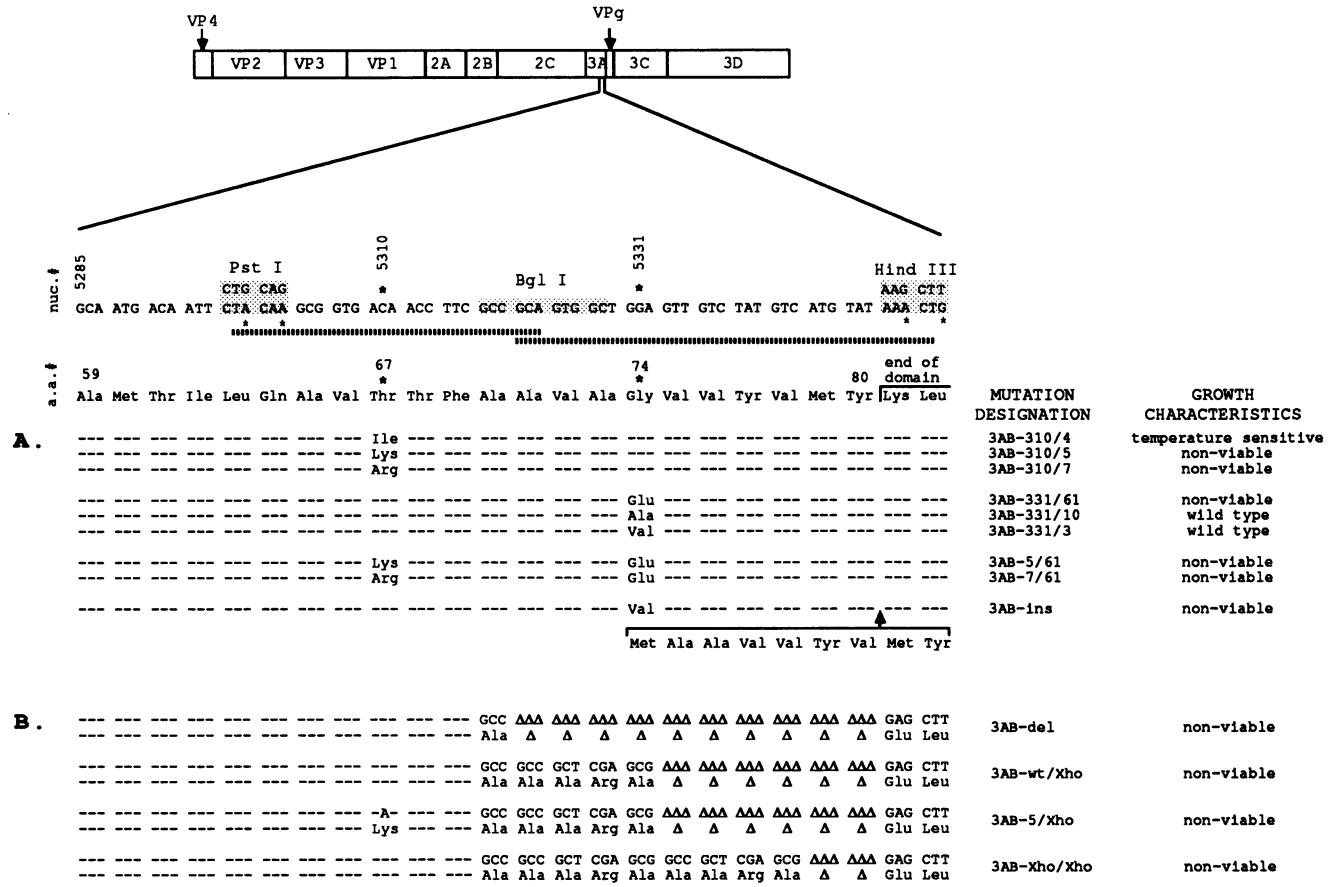

FIG. 1. Partial nucleotide (nuc.) and amino acid (a.a.) sequences of the hydrophobic domain of 3AB, showing the mutations described in this report and in a previous study (17, 18). The asterisks at nucleotides 5299, 5302, 5353, and 5356 denote the changes made by site-directed mutagenesis to introduce two new restriction sites: a *Pst*I site at position 5297 and a *Hin*dIII site at position 5351. (A) The broken line shows synthetic double-stranded oligonucleotides, used to introduce randomized nucleotides at positions 5310 and 5331, and a 9-amino-acid insertion between Tyr-80 and Lys-81. (B) Deletion and linker insertion mutants were made by digestion of wild-type or mutant 3AB-310/5 cDNAs with *Bgl*I and *Hin*dIII and then treatment with Klenow fragment and ligation in the presence or absence of *Xho*I linkers [5′-d(CCGCTCGAGCGG)-3′]. Δ, nucleotide or amino acid deletion.

complex isolated from PV-infected HeLa cells (56, 57). Since VPg contains polar and charged amino acid residues, it most likely requires a lipophilic carrier to provide its function in a membranous environment. It has been proposed that polypeptide 3AB, or perhaps any of the 3AB-containing polypeptides (e.g., P2-3AB or 2C-3AB), may act as a VPg donor during the replication process (53, 58). These polypeptides were found specifically associated with membrane fractions of PV-infected cells, presumably because they contain an uncharged sequence of 22 amino acids just upstream from VPg (53). Direct evidence for the role of this domain of 3AB and support for the above model were provided by our previous work in which we reported the isolation and analysis of a temperature-sensitive mutant containing a lesion in the hydrophobic domain of 3AB (mutant Se-1-3AB-310/4) (17). We showed that this mutation affects in vivo initiation of vRNA synthesis, in vitro uridylylation of VPg, and in vivo plus-strand RNA synthesis (18). To define the role of the hydrophobic domain of 3AB, we combined a genetic approach with an in vitro biochemical analysis that includes a membrane association assay with purified dog pancreas microsomes. We report the generation of substitution, deletion, and insertion mutations in the hydrophobic domain of PV polypeptide 3AB that further establish the role of this region in vRNA synthesis. Our data also suggest that the function(s) provided by 3AB cannot be provided in *trans*. We discuss the implications of these results on the formation of limited-diffusion replication complexes as a means of sequestering P2- and P3-region polypeptides required for RNA synthesis and protein processing.

MATERIALS AND METHODS

Generation of mutations in the hydrophobic domain of polypeptide 3AB. Two different approaches were used to generate mutations in the hydrophobic domain of polypeptide 3AB. Amino acid replacement mutations and the 9-amino-acid insertion mutant were created by cassette mutagenesis (Fig. 1A). The change of Thr-67 to Ile, Lys, or Arg has been described previously (17). To change Gly-74 to Glu, Ala, or Val, we first introduced a new *Hin*dIII restriction site at position 5351 using oligonucleotide-directed site-specific mutagenesis (22) of a subgenomic PV cDNA. One pair of synthetic oligonucleotides, corresponding to the region of

the PV genome between nucleotides 5321 and 5354 containing nucleotides randomized at position 5331, was inserted between the naturally occurring *Bgl*I site and the newly generated *Hin*dIII site. The 9-amino-acid insertion mutant was created by insertion of two mutagenesis cassettes between the *Bgl*I and *Hin*dIII sites. Putative mutations were screened by sequencing of double-stranded plasmid DNA (17). Double-amino-acid-change mutants were made by replacement of a *Bgl*I-to-*Bgl*II fragment containing the Gly-74-to-Glu mutation by the corresponding fragment of subgenomic PV cDNAs containing either the Thr-67-to-Lys change or the Thr-67-to-Arg change.

The deletion and the linker insertion mutations (Fig. 1B) were made by digestion of subgenomic PV wild-type or 3AB-310/5 mutant cDNAs with *Bgl*I and *Hin*dIII and then treatment with the Klenow fragment of DNA polymerase I. To make the 10-amino-acid deletion, the blunt-ended fragment was gel purified and its termini were joined by incubation with T4 ligase. To make the linker insertion mutations, the blunt-ended fragment was incubated with a phosphorylated *Xho*I linker, 5′-d(CCGCTCGAGCGG)-3′ (New England Biolabs) and T4 ligase. The excess *Xho*I linkers were removed by digestion with *Xho*I, and the linearized plasmids were gel purified before incubation with T4 DNA ligase. Positive clones were identified by the presence of an *Xho*I site in their corresponding plasmid DNA. Sequences were confirmed by sequencing of double-stranded plasmid DNA. Finally, a *Bgl*II (nucleotide 5601)-to-*Nsi*I (nucleotide 4830) fragment containing the specific changed sequence was used to replace the corresponding fragment of an infectious plasmid pPVA55 (31) and two transcription vectors, pT7-1 (64) and pT7-1(τ)PV1 (12).

cDNA transfections. Derivatives of plasmid pPVA55 containing specific lesions were transfected into HeLa cell monolayers at different temperatures by the calcium phosphate coprecipitation technique (21). Dilutions of the medium from mutant cDNA 3AB-310/4-transfected cells at 33°C or from mutant cDNAs 3AB-331/3 and 3AB-331/10 at 37°C were used to infect cells under semisolid agar. Well-isolated plaques were used to generate second-passage (P2) virus stocks. RNA was extracted from P2 mutant-infected cells, and the presence of the introduced mutation was confirmed by dideoxynucleotide sequence analysis with reverse transcriptase.

RNA transfections. RNA was synthesized in vitro from pT7-1(τ)PV1 or its derivatives containing 3AB lesions by using T7 RNA polymerase as described by Charini et al. (12). HeLa cells on 60-mm dishes were transfected with 0.05, 0.5, and 5 μg of in vitro-transcribed RNA by the DEAE-dextran procedure (34) with the modifications described previously (12). After transfection, cells were overlaid with liquid or semisolid agar medium and incubated at 33, 37, or 39°C, as noted in the text.

RNA extraction and slot-blot analysis. Total cytoplasmic RNA was extracted and denatured with glyoxal and dimethyl sulfoxide as previously described (18). After denaturation, RNA samples were diluted 10-fold with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and slot blotted onto GeneScreen Plus membranes (Du Pont). Membranes were prehybridized and hybridized to ^{32}P-labeled PV-specific synthetic oligonucleotides as described elsewhere (18).

In vitro translations and processing of protein samples. pT7-1-derived RNAs were translated in vitro at 30°C in a rabbit reticulocyte lysate supplemented with an uninfected HeLa cell extract (63). After 3 h of incubation, the reaction mixtures were diluted 10-fold in Laemmli sample buffer (LSB) (33), and the [^{35}S]methionine-labeled proteins were analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS). Gels were fluorographed and exposed to Kodak XAR film at −70°C.

In some cases, proteins produced by in vitro translation were immunoprecipitated with a rabbit antiserum directed against a bacterially expressed fusion 3AB protein (16b) by a previously described protocol (53). Immunoprecipitated 3AB and 3A proteins were subjected to electrophoresis on a 20% polyacrylamide, SDS-containing minigel (Bio-Rad), using a Laemmli gel buffer system with a separating gel of pH 8.

In vitro translations in the presence of microsomal membranes. In vitro translations of pT7-1-ΔNS-derived RNAs were done under the same conditions as described above for full-length RNAs, with the following modifications. The translation mix containing the rabbit reticulocyte lysate and the HeLa cell extract was centrifuged at 50,000 × *g* for 45 min to remove endogenous membranes before the addition of the viral RNA. Once the RNA was added, the total translation sample was divided into two 26.4-μl aliquots, and 3.6 μl of canine pancreatic microsomal membranes (Promega) in microsomal membrane buffer (50 mM triethanolamine, 2 mM dithiothreitol, 250 mM sucrose) or 3.6 μl of microsomal membrane buffer was added to each aliquot. After 3 h of incubation at 30°C, translation reaction mixtures were placed on ice. A 3-μl aliquot was diluted 10-fold with LSB and saved as the total reaction sample to control for the efficiency of the translation in the presence or absence of microsomal membranes. The remainder of the translation reaction mixtures were diluted twofold with physiological salt buffer (19) and layered over a 40-μl 0.5 M sucrose cushion in the same buffer. In vitro-translated proteins were separated into a membrane-bound (or pellet) fraction and a membrane-free (or supernatant) fraction by centrifugation at 4°C in a Beckman TL-100 tabletop ultracentrifuge with a TLA-100.3 fixed-angle rotor. Centrifugation was for 18 min at 35,000 rpm (approximately 50,000 × *g*). After centrifugation, the supernatant (including the sucrose cushion) was removed, diluted twofold with LSB, and saved. The pellet fraction was carefully washed three times with 100 μl of TE buffer (10 mM Tris [pH 8], 2 mM EDTA) and resuspended in 10 μl of LSB. Proteins present in the supernatant and pellet fractions were analyzed in 20% polyacrylamide, SDS-containing minigels as described above.

RESULTS

Generation of viable mutants and lethal mutations in the hydrophobic domain of polypeptide 3AB. Isolation and characterization of the temperature-sensitive mutant 3AB-310/4 have been previously described (17, 18). To isolate other single-amino-acid-replacement mutants, we transfected full-length plasmids (pPVA55 [31]) containing different 3AB mutations (310/5, 310/7, 331/61, 331/10, and 331/3 [Fig. 1A]), as well as wild-type PV cDNA, into HeLa cell monolayers at 33, 37, and 39°C. Transfection of HeLa cells with mutant cDNAs 3AB-331/3 and 3AB-331/10 (which specify Val and Ala at position 74, respectively, within 3AB) resulted in levels of virus production similar to those obtained after wild-type cDNA transfections at all the temperatures assayed. Plaque assays at 33°C versus 39°C showed similar reproductive capacity at both temperatures. The kinetics of RNA synthesis in cells infected with mutant viruses 3AB-331/10 and 3AB-331/3 at 33 or 39°C were similar to those

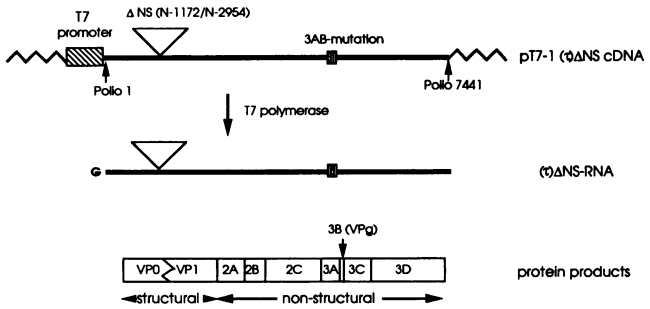

FIG. 2. Generation of mutant (τ)ΔNS RNAs. pT7-1(τ)ΔNS RNAs were constructed by deletion of the *Nru*I-*Sna*BI fragment from wild-type or mutant pT7-1(τ) full-length plasmid DNAs. pT7-1(τ) cDNA consists of a full-length copy of PV1(M) cDNA under control of the bacteriophage T7 promoter. The cloning scheme was designed so that only one extra nucleotide is present between the transcription initiation site and PV nucleotide 1 (12). In vitro transcription of pT7-1(τ)ΔNS RNAs with T7 polymerase generates vRNAs containing one extra guanosine residue at the 5′ end. Translation products generated from (τ)ΔNS RNAs are shown at the bottom of the figure.

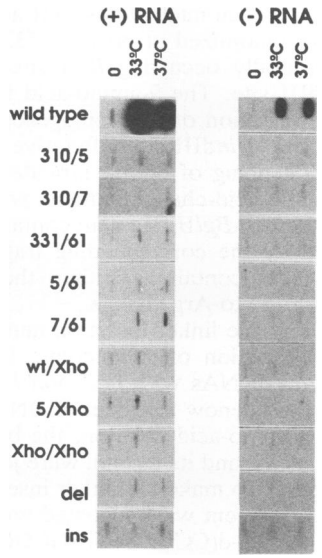

FIG. 3. Viral RNA accumulated in HeLa cells after transfections with 3AB mutant (τ)ΔNS RNAs. HeLa cells were transfected with 5 μg of in vitro-transcribed (τ)ΔNS RNAs. At 0 and 8 h posttransfection at 37°C, or 12 h posttransfection at 33°C, total intracellular RNA was extracted, denatured, and slot blotted onto membranes. Membranes were hybridized to ^{32}P-labeled PV-specific synthetic oligonucleotides designed to detect plus (+)- or minus (−)-strand vRNA.

observed for wild-type virus at these temperatures (data not shown). Therefore, we concluded that replacement of Gly-74 by Ala or Val has no significant effect on the growth and RNA synthesis capability of PV.

In contrast to the above results, transfection of HeLa cells with mutant cDNA 3AB-310/5, 3AB-310/7, or 3AB-331/61 (which specify for Lys or Arg at position 67 and Glu at position 74, respectively) failed to produce infectious virus at any of the temperatures assayed. Since RNA transfections are more sensitive than DNA transfections, the above 3AB mutations (310/5, 310/7, and 331/61) as well as the remaining mutations (the double-amino-acid-replacement mutations [3AB-5/61 and 3AB-7/61] [Fig. 1A], the 9-amino-acid insertion mutation [3AB-ins], the 10-amino-acid deletion mutation [3AB-del], and the linker insertion mutations [3AB-wt/Xho, 3AB-5/Xho, and 3AB-Xho/Xho] [Fig. 1B]) were cloned into a full-length T7 RNA expression vector, pT7-1(τ)PV1 (12). Transfection of HeLa cells with pT7-1(τ)PV1 mutant-derived RNAs failed to produce virus at 33, 37, or 39°C. In some cases (amino acid replacement mutations and insertion mutations), a few plaques developed after 5 to 7 days of incubation of the RNA-transfected cells at 33 or 37°C. Sequence analysis of RNA extracted from HeLa cells infected with P2 stocks derived from these plaque isolates showed that, in all cases, the mutant sequence had reverted to the original wild-type sequence. These results indicated that all the mutations are lethal for viral growth. Figure 1 summarizes the structure and infectivity characteristics of the mutations introduced in the hydrophobic domain of 3AB analyzed in this study.

Absence of vRNA accumulation in nonviable mutant-transfected cells. We were interested in determining the effects of the 3AB nonviable mutations on vRNA replication. To do so, we first generated the nonviable mutations in pT7-1(τ)ΔNS clones. As shown in Fig. 2, pT7-1(τ)ΔNS clones were constructed by deleting the *Nru*I (nucleotide 1172)-*Sna*BI (nucleotide 2954) PV fragment from mutant pT7-1(τ) full-length clones. This results in deletion of the carboxyl portion of VP0, all of VP3, and the amino-terminal portion of VP1. It has been previously demonstrated that the P1 deletion does not affect in vivo (27) or in vitro (64) proteolytic processing of P2- and P3-derived proteins. Therefore, the presence of normal P2 and P3 products allows wild-type pT7-1(τ)ΔNS transcripts to self-replicate after transfection in HeLa cells. In addition, the lack of normal P1 products apparently results in a more efficient RNA synthesis process: vRNA accumulates earlier and at higher levels in cells transfected with (τ)ΔNS RNAs compared with full-length RNA-transfected cells (16a, 27). Since transfection with (τ)ΔNS RNAs provides a sensitive method for detection of RNA synthesis after RNA transfection, we used this assay to study our nonviable mutants.

HeLa cells were transfected with pT7-1(τ)ΔNS mutant- or wild-type-derived RNAs. At 8 h posttransfection at 37°C or 12 h posttransfection at 33°C, total intracellular RNA was extracted, blotted to nylon membranes, and probed for the presence of plus- or minus-strand vRNA. The harvesting times selected correspond to maximum levels of vRNA accumulation in wild-type (τ)ΔNS RNA-transfected cells (data not shown). Analysis of the data shown in Fig. 3, in which one representative experiment is presented, indicates that none of the mutant (τ)ΔNS RNA-transfected cells were able to accumulate normal levels of plus-strand or minus-strand vRNAs at 33 or 37°C. In some experiments (as shown here, for mutant 7/61), very low levels of vRNA were detected in cells transfected with (τ)ΔNS RNAs containing the amino acid replacement mutations. Since this result was not reproducible, we speculate that the small amount of RNA synthesis found in these cases was likely due to the variable presence of small numbers of revertants in the population. Our results indicated that all the nonviable mutations introduced in 3AB affected viral RNA production.

In vitro translation of RNAs containing 3AB mutations. Since we found that all the nonviable mutations introduced in 3AB have an effect on vRNA synthesis, it was important to determine whether these were primary defects or defects that resulted from impaired proteolytic processing. To study the protein synthesis and protein-processing capability di-

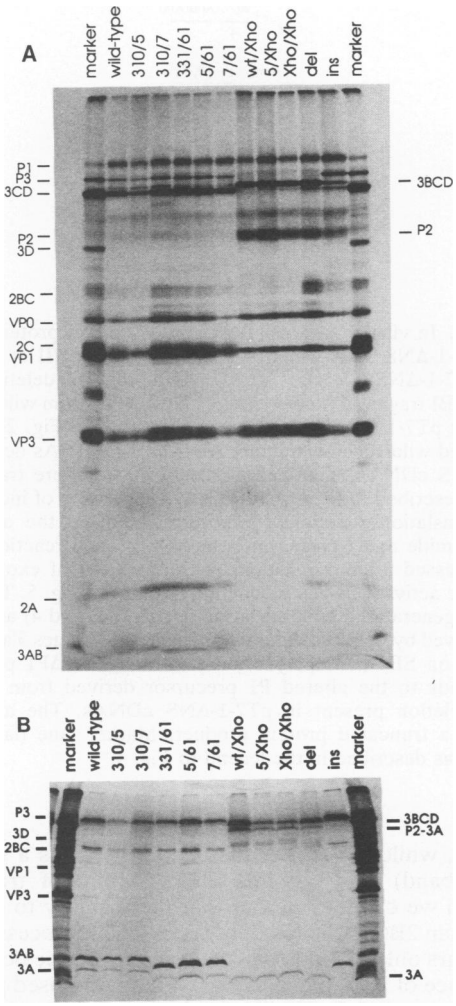

FIG. 4. (A) In vitro protein synthesis directed by wild-type and 3AB mutant RNAs. In vitro-transcribed wild-type or mutant full-length RNAs were translated for 3 h in rabbit reticulocyte lysates supplemented with uninfected HeLa extract. After the translation, the reaction mixtures were diluted with LSB and analyzed on an SDS-12.5% polyacrylamide gel. Marker lane, [^{35}S]methionine-labeled PV proteins isolated from an extract of infected HeLa cells. (B) In vitro-translated proteins from panel A were subjected to immunoprecipitation with anti-3AB serum and analyzed on a 20% polyacrylamide, SDS-containing minigel.

rected by RNAs containing the 3AB lesions, we performed in vitro translations of full-length pT7-1-derived RNAs and then immunoprecipitated the products of translation with α3AB antiserum. Analysis of the data presented in Fig. 4 indicated that different protein processing patterns were found after translation of different mutant RNAs. Transcripts derived from single- or double-amino-acid-replacement mutants (3AB-310/5, 3AB-310/7, 3AB-331/61, 3AB-5/61, and 3AB-7/61) gave wild-type protein processing patterns. The only difference we found was a different mobility in SDS-polyacrylamide gels of the mutated 3A and 3AB proteins of mutants 3AB-331/61, 3AB-5/61, and 3AB-7/61 (Fig. 4B). These data suggest that the single- or double-amino-acid mutations made in 3AB primarily affected the role of this polypeptide in vRNA synthesis.

In contrast to amino acid replacement mutations, all the other mutations, in which the number of amino acids of 3AB was altered, affected the normal processing of the polyprotein. The translation pattern of the linker insertion mutants (3AB-wt/Xho, 3AB-5/Xho, and 3AB-Xho/Xho) showed abnormal P2 and P3 processing. Polypeptide P3 was primarily cleaved to 3A plus 3BCD, so that barely detectable amounts of 3CD and 3AB were formed, and most of the P2 polypeptide remained unprocessed (Fig. 4A). Finally, low levels of an abnormal product, P2-3A (identified by immunoprecipitation with α2C, α3A, α3B [data not shown], and α3AB [Fig. 4B] antibodies), were found. The translation and processing patterns of RNAs encoding the 9-amino-acid insertion (3AB-ins) and 10-amino-acid deletion (3AB-del) mutants were somewhat intermediate between the normal and the linker insertion mutant patterns. For these lesions, P2 was partially processed, so that some 2BC and 2A was found, and in the case of 3AB-ins, less 3BCD was produced compared with the other insertion and deletion mutations (Fig. 4A). Collectively, the in vitro translation and processing data from this second class of mutations suggested that the inability to produce virus by cDNA clones bearing amino acid insertions or deletions in 3AB was related to a primary defect in protein processing.

P2 polypeptide is not a major precursor in PV proteolytic processing. The lack of normal P2 (and 2BC) processing in mutants 3AB-wt/Xho, 3AB-5/Xho, 3AB-Xho/Xho, 3AB-del, and 3AB-ins was rather surprising, since this precursor contained no alterations and, in all cases, the P1 precursor was processed very efficiently, indicating the presence of normal 3C/3CD proteinase activity. To determine whether the inefficient P2 processing was the result of an insufficient 3C proteolytic activity during the in vitro translations due to the abnormal P3 processing, we analyzed protein processing after the addition of exogenous proteinase activity. To do so, mutant RNAs were translated in vitro, as described above, and after 3 h of translation, the translation reactions were terminated by the addition of cycloheximide and RNase. A PV type 1 (PV1)-infected HeLa cell extract containing virus-specific proteinases was then added to a portion of the translation mixture, and after 2 h of incubation at 30°C, the products of the translation were analyzed in SDS-polyacrylamide minigels. Addition of PV proteinases to 3AB-del and 3AB-Xho/Xho mutant translation reactions resulted in further processing of polypeptide 3BCD into 3CD (Fig. 5, lanes 4 versus 5 and 6 versus 7). However, no increase in the amount of 2BC or 2C was observed, demonstrating that P2 or 2BC was not processed further. Similar results were obtained when the other mutants were analyzed (data not shown). Two different explanations may account for this result. (i) P2 cannot be processed in *trans*. This suggests that the formation of P2 products occurs via 3C *cis* cleavages, perhaps by a mechanism similar to the one proposed for other picornaviruses (23, 24, 44, 50). (ii) Or, rather than a *cis* versus *trans* activity encoded by 3C, P2 may not be an appropriate substrate for 3C. This would implicate that the actual precursor for polypeptides 2A, 2BC, 2B, and 2C is a P2-P3-containing polypeptide (i.e., P2-P3, P2-3ABC, P2-3AB, or P2-3A).

To distinguish between the two possibilities mentioned above, we artificially created a P2-3ABC* precursor (* indicates a truncated 3C protein) to serve as the substrate for 3C in a posttranslational processing assay similar to the one described in the legend to Fig. 5. Moreover, to be able to better analyze the production of P2 and P3 products, we introduced a P1 deletion, from nucleotide 1172 to nucleotide 2954, in the cDNAs (pT7-1-ΔNS clone [64] [Fig. 2]). By

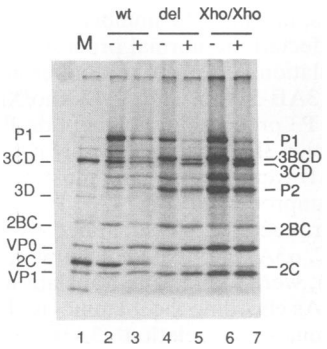

FIG. 5. In vitro posttranslational processing of wild-type (wt) and mutant protein products by exogenous 3C proteinase activity. In vitro-transcribed wild-type or mutant full-length RNAs were translated in vitro as described in the legend to Fig. 4. The translation reactions were terminated by the addition of cycloheximide and pancreatic RNase to 5 and 10 μg/μl, respectively. After 10 min of incubation at 30°C, each reaction mixture was divided into two 10-μl aliquots, and 3 μl of lysis buffer used for extract preparation (−, lanes 2, 4, and 6) or 3 μl of PV-infected HeLa extract (+, lanes 3, 5, and 7) was added. After 2 h of incubation at 30°C, translation reaction mixtures were diluted with LSB and analyzed on an SDS-7.5% polyacrylamide minigel. Marker lane (lane 1) was prepared as described in the legend to Fig. 4.

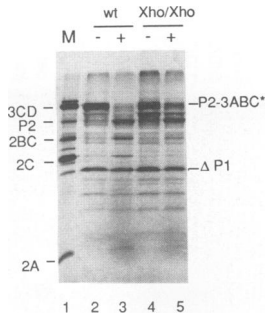

FIG. 6. In vitro posttranslational processing of proteins derived from pT7-1-ΔNS cDNA clones linearized with *Bgl*II at nucleotide 5601. pT7-1-ΔNS cDNAs were constructed by deletion of the *Nru*I-*Sna*BI fragment (nucleotides 1172 to 2954) from wild-type (wt) or mutant pT7-1 full-length plasmid cDNAs (64) (Fig. 2). In vitro-transcribed wild-type or mutant 3AB-Xho/Xho RNAs derived from pT7-1-ΔNS cDNAs linearized at the *Bgl*II site were translated in vitro as described in the legend to Fig. 4. After 3 h of incubation at 30°C, translation reactions were terminated by the addition of cycloheximide and RNase, and one-half of each reaction mixture was processed after translation by the addition of exogenous 3C proteinase activity, as described in the legend to Fig. 5. The protein products generated after translation (−, lanes 2 and 4) and translation followed by posttranslational processing (+, lanes 3 and 5) were analyzed on SDS-15% polyacrylamide minigels. ΔP1 polypeptide corresponds to the altered P1 precursor derived from the *Nru*I-*Sna*BI deletion present in pT7-1-ΔNS cDNAs. The asterisk (*) indicates a truncated protein product. Marker lane (lane 1) was prepared as described in the legend to Fig. 4.

linearization of the wild-type or the mutated ΔNS cDNAs at nucleotide 5601, the polyproteins generated by translation of the RNA transcripts derived from these truncated cDNAs would not have 3C proteinase activity. In vitro translation of these wild-type or mutant ΔNS RNAs generated two products: a ΔP1 polypeptide of about 30 kDa (produced by 2A processing at the P1-P2 cleavage site) and a P2-3ABC* polypeptide of about 100 kDa (Fig. 6, lanes 2 and 4). When posttranslational processing of the wild-type precursor was assayed by the addition of a PV1-infected HeLa cell extract, as a source of 3C, processing of P2-3ABC* into P2, 2A, 2BC, and 2C was observed (Fig. 6, lane 3). This confirmed that the proteinase is able to make these cleavages in *trans*. On the contrary, addition of exogenous 3C proteinase activity to the P2-3ABC* precursor containing the 3AB-Xho/Xho mutation resulted mostly in P2 formation, with little 2A or 2BC production and no detectable 2C production (Fig. 6, lane 5). A similar result was observed when mutations 3AB-wt/Xho and 3AB-5/Xho were analyzed (data not shown). One likely explanation for this result is that the change in the number of amino acids at the carboxyl terminus of 3AB produced a misfolding of the polypeptide chain around the P2-P3 cleavage site. This conformational change affected the accessibility and the efficiency of $3C^{pro}$ cleavage at the different cleavage sites so the processing order of P2-P3 was altered. This alteration may be responsible for the abnormal P2 processing of these mutants.

To further study the order of the proteolytic processing, we analyzed the in vitro kinetics of protein synthesis and processing for wild-type and mutant 3AB-Xho/Xho. To do so, we translated mutant or wild-type ΔNS RNAs in vitro, and at different times after translation, aliquots were removed and the proteins present at each time point were analyzed. As shown in Fig. 7 for wild-type ΔNS RNA translations, polypeptides 2BC and 2C were detected after 20 min of translation, while P2 detection required 25 to 30 min. A longer exposure of the autoradiogram shown in Fig. 7 indicated that after 15 min of translation, 2BC was clearly detected, while the earliest detection of P2 (as a very faint protein band) was possible after 20 min of translation. Although we cannot rule out that the inability to detect P2 earlier than 2BC is the result of a very rapid processing of P2 that occurs only early during the translation, the delay in the appearance of P2 is inconsistent with its proposed precursor role for 2A and 2BC. Similar results were obtained when the kinetics of protein production induced by full-length wild-type RNA transcripts were analyzed (data not shown).

In contrast to the data obtained for wild-type ΔNS translation and processing, analysis of the kinetics of translation of mutation 3AB-Xho/Xho showed that polypeptide P2 was clearly present after 20 min of translation, while detection of 2BC and 2C required 25 and 30 min, respectively. This indicated that the kinetics of P2 processing of the 3AB mutant are different from the wild-type kinetics, confirming that the order of cleavages in the mutated P2-P3 precursor is altered. Apparently, the change in the number of amino acids near the carboxyl terminus of 3A exposes the 3A-3B and 2C-3A cleavage sites so that the proteinase 3C cleaves them first. As a result of these early cleavages within the P2-P3 precursor, two of the major final nonstructural protein products of translation of mutants 3AB-wt/Xho, 3AB-5/Xho, and 3AB-Xho/Xho were 3BCD and P2, instead of 3CD, 3AB, and the P2-derived protein products. Interestingly, a bigger change in the number of inserted or deleted amino acids, as for the 3AB-del or 3AB-ins mutations (in which 10 amino acids were deleted or 9 amino acids were inserted), partially corrected the processing defect, since in these cases polypeptides 3CD and 2BC were formed (Fig. 4A).

Mutated 3AB proteins exhibit normal in vitro association with microsomal membranes. Proteins 3A and 3AB have been found associated with the membranes of PV infected cells (53, 58, 59), probably due to the presence of a 22-amino-

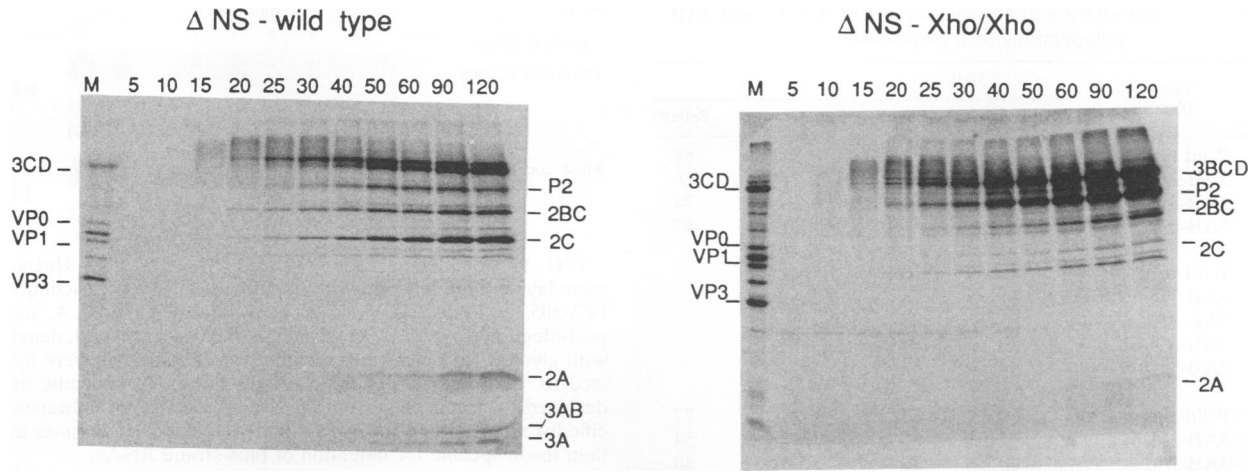

FIG. 7. In vitro kinetics of protein synthesis and processing directed by wild-type or mutant 3AB-Xho/Xho ΔNS RNAs. In vitro-transcribed wild-type or mutant ΔNS RNAs were translated in vitro by using the conditions described in the legend to Fig. 4. At the times (minutes) indicated in the figure, aliquots from each translation reaction mixture were withdrawn, and translation was terminated by dilution of the aliquots with LSB. Translation products generated at each time point were analyzed on SDS-15% polyacrylamide minigels. M, marker lane.

acid hydrophobic domain (53). It has been proposed that 3AB acts as a lipophilic carrier for VPg to initiate vRNA synthesis (53, 58). Since all the amino acid replacement mutants that introduced charges in the hydrophobic domain of 3AB have a defect in vRNA synthesis, it was of interest to determine whether this defect correlated with an abnormal association of the mutated proteins with membranes. The 3AB mutations (described above) resulted in nonviable constructs and were unable to synthesize detectable levels of viral macromolecules after transfection in HeLa cells (unpublished data). Therefore, we developed an in vitro system to study protein-membrane interactions. We selected an in vitro translation assay in the presence of canine pancreatic microsomal membranes. This system has been used extensively to study the mechanism of insertion of proteins in cellular membranes (1, 41, 55, 62), translocation of proteins across membranes (19), and protein-membrane interactions in other viral systems (51), but it has not been previously used to study PV protein-membrane associations.

To study the in vitro association of PV proteins with membranes, we translated pT7-1-ΔNS-derived wild-type or mutant RNAs in the presence or absence of microsomal membranes. After the translation, the in vitro-synthesized proteins were separated by ultracentrifugation into a membrane-bound (or pellet) fraction and a membrane-free (or supernatant) fraction, and the proteins present in each fraction were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 8, in which one representative experiment is displayed, the pellet fractions from translations made in the absence of membranes showed the presence of variable amounts of nonspecifically precipitated viral proteins (Fig. 8A). Therefore, we based our further analysis on quantitation of only the proteins present in the supernatant fractions. With polypeptides 3A and 3AB, we

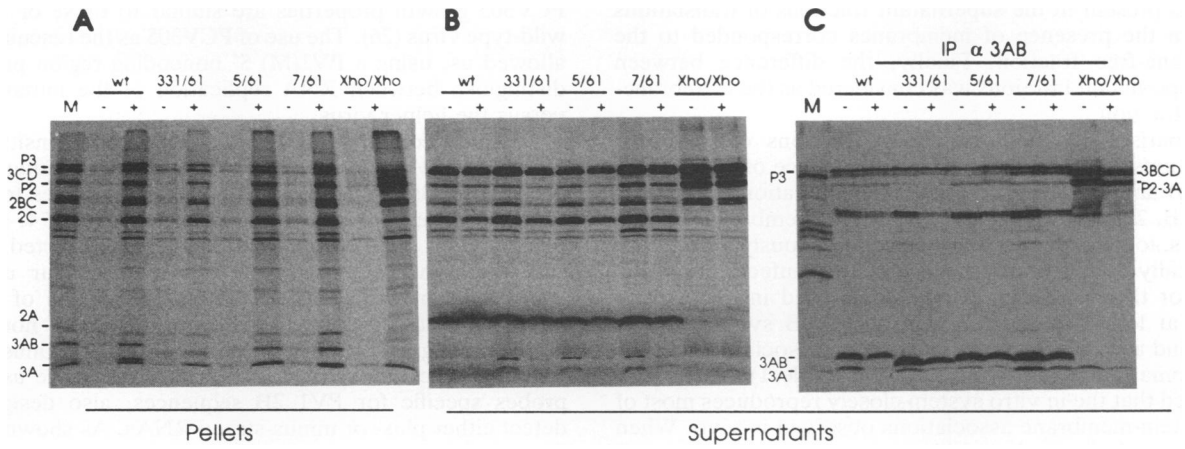

FIG. 8. Association of mutated 3AB and 3A proteins with membranes. Mutant and wild-type (wt) pT7-1-ΔNS RNAs were translated in vitro in the presence (+) or absence (−) of canine pancreatic microsomal membranes. The products of the translations were fractionated on sucrose step gradients into pellet (membrane-associated) (A) or supernatant (membrane-free) (B) fractions and analyzed on SDS-20% polyacrylamide minigels. Proteins present in supernatant fractions were immunoprecipitated with an anti-3AB serum before SDS-PAGE analysis (C). M, marker lane.

TABLE 1. Quantitation of association of mutated 3A and 3AB polypeptides with membranes[a]

Expt	Virus or plasmid	3AB		3A	
		Supernatant[b]	Pellet[c]	Supernatant[b]	Pellet[c]
1	Wild type	38		32	68
	3AB-310/5	45	55	34	66
	3AB-310/7	32	68	26	74
	3AB-ins			53	47
2	Wild type	40	60		
	3AB-310/5	57	43		
	3AB-310/7	55	45		
	3AB-331/61	20	80		
	3AB-5/61	58	42		
3	Wild type	50	50	23	77
	3AB-331/61	34	66	46	54
	3AB-5/61	60	40	60	40
	3AB-7/61	41	59	36	64
	3AB-Xho/Xho			66	34

[a] Proteins translated in vitro in the presence or absence of microsomal membranes were separated into membrane-bound or pellet and membrane-free or supernatant fractions, as described in the legend to Fig. 5. After the translation, 3A and 3AB proteins present in supernatant fractions were immunoprecipitated with an anti-3AB serum, separated by SDS-PAGE, and quantitated by using a laser densitometer (LKB UltroScan XL). Results from three independent experiments are shown.

[b] Percentages of the amount of 3A and 3AB proteins present in the supernatant fractions of translations made in the presence of membranes. For the calculation, it was considered that the total (100%) of 3A or 3AB protein synthesized in vitro was the amount of protein present in the supernatant fractions of translations made in the absence of membranes.

[c] Percentages of the amount of 3A and 3AB proteins present in the pellet or membrane-bound fraction. The value obtained is the difference between the total amount of protein synthesized (3A or 3AB protein present in the supernatant fraction of translations made in the absence of membranes) and the amount of protein present in the membrane-free fraction (3A or 3AB proteins present in the supernatant fraction of translations made in the presence of membranes).

used laser densitometric scanning to quantitate the proteins present in the supernatant fractions after immunoprecipitations with α3AB antiserum. We assumed that the proteins present in the supernatant fractions of translations made in the absence of membranes corresponded to the total amount of protein synthesized in vitro and that the amount of proteins present in the supernatant fractions of translations made in the presence of membranes corresponded to the membrane-free fraction. Finally, the difference between both supernatant fractions was considered as the membrane-bound fraction.

Comparison of the supernatant fractions of wild-type translations made in the presence or absence of membranes (Fig. 8B and C) indicated a specific association of proteins 3A, 3AB, 2C, and 2BC with microsomal membranes. These proteins, together with 3D, have been previously found to be specifically bound to membranes of cells infected with PV (59). For the experimental conditions used in the present study, at least 50% of the wild-type 3AB synthesized in vitro, and about 70% of 3A, were found associated with the microsomal membranes (Fig. 8C and Table 1). This result indicated that the in vitro system closely reproduces most of the protein-membrane associations observed in vivo. When we analyzed the amino acid replacement mutations, we found that the distribution of the different mutated 3A and 3AB proteins between the membrane-bound or pellet fraction and the free or supernatant fraction was very similar to the distribution obtained for the wild-type proteins. As

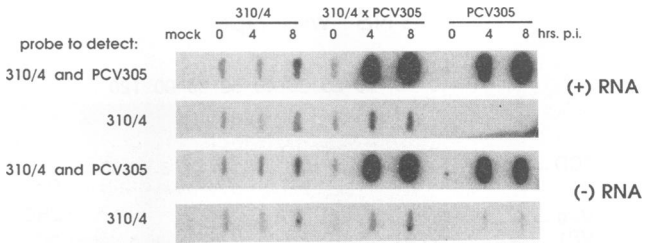

FIG. 9. Complementation of mutant 3AB-310/4. HeLa cell monolayers were infected with mutant 3AB-310/4 or rescuing virus PCV305 or coinfected with both viruses at 39°C. At 0, 4, and 8 h postinfection (hrs. p.i.), cytoplasmic RNA was extracted, denatured with glyoxal, and blotted to membranes. Membranes were hybridized to ^{32}P-labeled PV-specific probes or to 310/4-specific probes designed to detect plus- or minus-strand vRNAs. Membranes specific for minus-strand RNA detection were exposed 25 times longer than those specific for detection of plus-strand RNAs.

shown in Table 1 (in which three different experiments are compared), the differences we found were within the limits of the experimental error. These data suggested that the absolute in vitro association of 3A and 3AB polypeptides with membranes was not altered by the presence of one or two charged amino acids in the hydrophobic domain contained in this polypeptide. On the other hand, it appears that the introduction of more-dramatic changes in 3AB, as for 3AB-ins and 3AB-Xho/Xho, produced a reduced association of 3A with membranes (Fig. 8 and Table 1, experiments 1 and 3).

Viral replicative function altered by 3AB mutations cannot be complemented. Genetic complementation among different PV mutants has been studied by many groups as a tool to define *cis* versus *trans*-acting functions within the viral genome (5, 12, 14, 36, 52). We were interested in determining whether the RNA synthesis defect of the mutants we made in the hydrophobic domain of 3AB could be rescued in vivo by wild-type proteins provided in *trans*. We studied complementation of the temperature-sensitive mutant 3AB-310/4 and the nonviable mutations. As a rescuing virus, we used PCV305 (26), a PV-coxsackievirus recombinant virus in which the 5′ noncoding region of PV1(M) was replaced by the corresponding 5′ noncoding region of coxsackievirus B3. PCV305 growth properties are similar to those of PV1(M) wild-type virus (26). The use of PCV305 as the rescuing virus allowed us, using a PV1(M) 5′ noncoding region probe, to distinguish between RNA replication of the mutant virus versus the helper virus.

To study complementation of temperature-sensitive mutant 3AB-310/4, we infected HeLa cells with mutant 3AB-310/4, the helper virus (PCV305), or both viruses together. Infected cultures were incubated at 39°C. At 0, 4, and 8 h postinfection, total cytoplasmic RNA was extracted, immobilized on membranes, and hybridized to four different PV-specific probes. To detect RNA replication of mutant virus, we used two probes specific for the PV1 5′ noncoding region, designed to detect either plus- or minus-strand RNAs. To detect replication of both viruses, we used two probes specific for PV1 2B sequences, also designed to detect either plus- or minus-strand RNAs. As shown in Fig. 9, negligible amounts of plus- or minus-strand vRNA were detected in cells infected with mutant 3AB-310/4. In cells infected with mutant and helper virus, we observed an approximately threefold increase in the amount of plus- and minus-strand 3AB-310/4 RNA accumulated, suggesting that

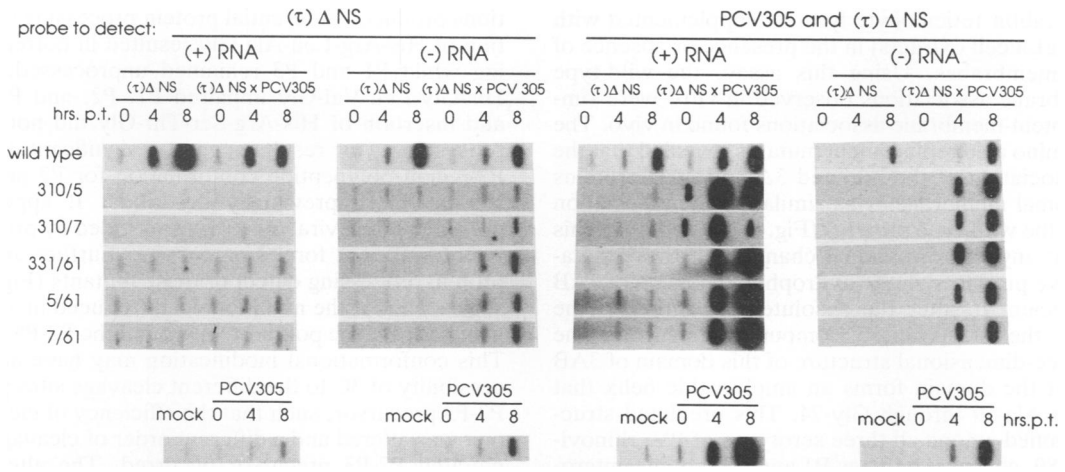

FIG. 10. Complementation of mutant (τ)ΔNS RNAs. HeLa cell monolayers were mock infected or infected with rescuing virus PCV305. One hour after infection, part of the infected cultures and part of the mock-infected cultures were transfected with mutant (τ)ΔNS RNAs. At 0, 4, and 8 h posttransfection (hrs. p.t.), total intracellular RNA was extracted, denatured with glyoxal, and blotted to membranes. The blots were hybridized to PV-specific probes or to ΔNS-specific probes designed to detect plus- or minus-strand RNAs. Membranes specific for detection of minus-strand RNAs were exposed 10 times longer than those specific for detection of plus-strand RNAs.

some complementation occurred. However, the amount of mutant RNA synthesis detected (as determined by laser densitometry) was still 60 to 80 times lower than the level of RNA synthesis found in the wild-type virus infection at 39°C or the amount of RNA synthesized by the mutant at the permissive temperature (data not shown). This indicated that the RNA synthesis defect of mutant 3AB-310/4 could not be efficiently rescued by providing wild-type proteins in *trans*.

To assay for complementation of the RNA synthesis defect of the constructs containing nonviable 3AB lesions, we used a slightly different approach. HeLa cell monolayers were mock infected or infected with the helper virus, PCV305. One hour after infection, part of the infected cultures and part of the mock-infected cultures (as a control for mutant RNA replication) were transfected with 5 μg of mutant (τ)ΔNS RNAs. Cells were incubated at 37°C, and at 0, 4, and 8 h after transfection, total intracellular RNA was extracted. Samples were analyzed for the presence of plus- or minus-strand vRNA corresponding to PCV305 or mutant RNA replication, as described above. Figure 10 shows the result of one complementation experiment, in which the amino acid replacement mutants were analyzed. As expected, no RNA accumulated in cells transfected with mutant (τ)ΔNS RNAs alone. In some cases (as shown here for mutants 3AB-5/61 and 3AB-7/61), we observed a very slight increase in the amount of plus-strand RNA accumulated in the cells that were infected with PCV305 before the mutant RNA transfection. This suggested that in some cases, a very low level of complementation occurred. But, as for the temperature-sensitive mutant 3AB-310/4, complementation was very inefficient.

DISCUSSION

In this study, we analyzed the effects on PV replication of several mutations engineered in the hydrophobic domain of polypeptide 3AB. The mutations we generated fell into two categories. Within the first group are the amino acid replacement mutations in which Thr-67 was changed to Lys, Arg, or Ile (mutations 3AB-310/5, 3AB-310/7, and 3AB-310/4, respectively) or in which Gly-74 was changed to Glu, Ala, or Val (mutations 3AB-331/61, 3AB-331/10, and 3AB-331/3, respectively) and two double mutations carrying the changes Gly-74 to Glu and Thr-67 to Lys or Arg (mutations 3AB-5/61 and 3AB-7/61, respectively) (Fig. 1A). The second group includes the mutations in which the number of amino acids of the hydrophobic domain between Ala-70 and Leu-82 was changed by introducing insertions, or deletions, or deletions followed by amino acid replacements (mutations 3AB-ins, 3AB-del, 3AB-wt/Xho, 3AB-5/Xho, and 3AB-Xho/Xho) (Fig. 1B).

In the first group, the change of Thr-67 to Ile produced a temperature-sensitive virus, which has been described previously (18). Replacement of Gly-74 by Ala or Val resulted in mutant viruses with no significant phenotypic alterations. In contrast, all the mutations that introduced one or two charges in the hydrophobic domain were nonviable (Fig. 1). RNAs containing these lethal mutations were unable to replicate after transfection into HeLa cells (Fig. 3). In addition, in vitro translation of these RNAs produced normal protein processing patterns (Fig. 4A). The above results suggested that the mutations we engineered in the hydrophobic domain of 3AB primarily affected vRNA production. These data are in agreement with our previous results with mutant 3AB-310/4 (18), indicating that this domain of 3AB participates in vRNA synthesis.

All the mutations that introduced charges diminished the average hydropathy value of the hydrophobic domain of polypeptide 3AB. Using the Kyte and Doolittle (32) hydropathy scale, we calculated (for a 17-amino-acid span, extending from Ile-62 to Val-78) values of H(17) = 1.92 for wild-type virus, H(17) = 1.57 for mutation 3AB-5/61, and H(17) = 1.51 for mutation 3AB-7/61. These values, when applied to the quadratic equation described by Klein et al. (28), predict that the proteins carrying such mutations will exhibit a peripheral membrane localization, rather than the internal (integral) localization expected for the wild-type protein. Therefore, it was of interest to experimentally test whether the mutated proteins could associate with membranes. To do so, we first developed an in vitro assay in which in vitro-synthesized wild-type or mutant vRNAs were

translated in rabbit reticulocyte lysates (supplemented with uninfected HeLa cell extracts) in the presence or absence of microsomal membranes. Using this assay, the wild-type protein-membrane associations observed in vitro were similar to the protein-membrane associations found in vivo. The analysis of amino acid replacement mutants revealed that the extent of association of the mutated 3A and 3AB proteins with microsomal membranes was similar to the association observed for the wild-type proteins (Fig. 8 and Table 1). This indicated that any conformational change that these mutations may have produced in the hydrophobic domain of 3AB was not sufficient to alter the absolute association of the protein with the membranes. Computer analysis of the predicted three-dimensional structure of this domain of 3AB indicated that the domain forms an amphipathic helix that extends from Ala-59 through Gly-74. This predicted structure is maintained among all three serotypes of PV, rhinoviruses 14 and 89, coxsackieviruses B1 and B3, bovine enterovirus, and swine vesicular disease virus UKG/27/72, independent of the amino acid heterogeneity present in the different viruses (20a). According to the structural model, Thr-67 lies on the hydrophilic side of the helix, perhaps interacting with another viral or cellular membrane protein rather than with cellular membrane phospholipids. Such a model may explain why the replacement of Thr-67 for Lys or Arg did not produce a clear change in the association of 3AB with microsomal membranes. In agreement with this model is the phenotype of mutant 3AB-310/4, in which the change of Thr-67 for Ile (that clearly would alter the amphipathicity of the presumed helix) generated a temperature-sensitive phenotype. Confirmation of this hypothesis will require mutation of amino acids located on the hydrophobic side of the helix and then analysis of the resulting phenotypes. Alternatively, it may be that the hydrophobic domain of 3AB, rather than providing a simple membrane anchor for 3AB, actually possesses another function, such as taking part in a specific interaction with another viral or cellular protein required for vRNA synthesis. Alteration of the hydrophobicity of the domain would modify such protein-protein interaction, which could result in an abnormal RNA synthesis.

The second group of mutations, in which the number of amino acids of the hydrophobic domain was altered, showed, in addition to the RNA synthesis defect (Fig. 3), protein processing defects (Fig. 4). The protein processing defects involved P2 and P3 polypeptide proteins. P3 was mainly cleaved into 3BCD and 3A, so that very little or no 3AB was formed. In addition, polypeptide P2 was processed very inefficiently. Thus, the lack of RNA synthesis displayed by these mutant constructs could be due to the lack of viral proteins necessary for RNA replication rather than to the presence of a mutated 3AB protein.

The lack of processing of polypeptide P2 exhibited by proteins derived from RNAs containing the 3AB insertions and deletions was rather unexpected since they were able to efficiently process polypeptide P1, thereby confirming the presence of an active viral proteinase. Although this result underscored previous data which demonstrated that the requirements for P2 processing are different from those required for P1 processing, it differed from previous protein processing studies which indicated that P1-processing determinants are more susceptible to perturbation than those of P2 processing (35). For example, Kuhn et al. (29) described linker insertion mutants in which three different groups of 5 amino acids were introduced near the carboxyl terminus of polypeptide 3A, between Gly-85 and His-86. These mutations produced differential protein processing defects. Insertion of His-Arg-Leu-Ala-Gly resulted in normal P2 processing while P1 and P3 remained unprocessed. Insertion of His-Gly-Leu-Val-Gly impaired P1, P2, and P3 processing, and insertion of His-Arg-Ser-Thr-Gly did not alter protein processing. Our results suggest a significant dependence of P3-region polypeptide conformation for P2 processing that has not been previously described. It appears that the presence of the viral proteinase provided by processing of P3 is not sufficient for P2 processing. Further analysis of the protein processing defect of these mutants (Fig. 5, 6, and 7) suggested that the mutation we introduced in 3A produced a misfolding of the polyprotein around the P2-P3 cleavage site. This conformational modification may have altered the accessibility of 3C to the different cleavage sites present in the P2-P3 precursor, such that the efficiency of cleavage at each pair was altered and a different order of cleavages within the mutated P2-P3 precursor occurred. The alteration of the normal order of 3C processing ultimately resulted in the abnormal protein processing pattern that we observed. Our results show that once P2 is formed (as the result of 3C activity at the 2C-3A cleavage site), it cannot be efficiently processed. This indicates that P2 is not the major precursor for polypeptides 2A and 2BC. In addition, the very inefficient posttranslational processing of 2BC into 2C and 2B that we observed with mutants 3AB-Xho/Xho (Fig. 5, lane 6 versus lane 7) and 3AB-del (Fig. 5, lane 4 versus lane 5) suggests that 2BC is not the actual precursor for polypeptides 2B and 2C. Interestingly, it was demonstrated that the relative efficiency of cleavage of synthetic 16-residue peptides corresponding to cleavage sites present in the P2 and P3 regions of the genome correlated roughly to the half-life of PV precursors in vivo (43). This *trans* cleavage assay suggested that the structural features residing within the 16-amino-acid span surrounding the cleavage site contribute in determining the order in which processing events occur (43). In addition, these same in vitro studies indicated that the 16-residue synthetic peptides corresponding to the 2C/3A and to the 2B/2C cleavage sites in the PV2 polyprotein were efficient substrates for in vitro cleavage by purified 3C when compared with peptides corresponding to the other cleavage sites present in the P2 and P3 precursors (43). However, note that the 2B/2C cleavage site in PV2 contains an alanine residue in the P4 position, whereas the corresponding site in PV1 (used in the present study) contains a valine residue in the P4 position. Since the P4 position has been shown to strongly influence the efficiency of 3C cleavage site utilization (8), extrapolation of cleavage data from sites containing different P4 amino acids may produce limited conclusions. The abnormal protein processing of our 3A mutants strongly suggested that the tertiary structure of the polyprotein plays a significant role in regulating the efficiency of 3C activity at each site.

Our results allow us to propose that the major early cleavages within the P2-P3 precursor involve the concomitant cleavages between 2A-2B and 2C-P3 to generate polypeptides 2A, 2BC, and P3, followed by processing of 2BC and P3. Other alternative processing pathways of the P2-P3 precursor may result from an initial cleavage within P3, perhaps at the 3B-3C cleavage site, to generate polypeptides P2-3AB plus 3CD, followed by processing of P2-3AB to yield polypeptides 2A plus 2BC-3AB, and followed by processing of 2BC-3AB to yield the final products 2B, 2C, and 3AB. These cleavage pathways are probably not based on an absolute, sequential order of cleavage events. Instead, the sequence of events is dictated by the relative frequency of

3C-mediated cleavage at a specific Q-G pair. Cleavage frequencies at individual sites may vary depending on the nature of the substrate (i.e., tertiary structure of large versus intermediate-sized precursors) and the nature of the enzyme-substrate interaction (i.e., *cis* versus *trans* reactions). Thus, an initial cleavage at the 2A-2B junction occurs very rapidly in a *cis* or *cis*-like fashion within the P2-P3 precursor polypeptide. This same cleavage event occurs much more slowly as a *trans* event after cleavage at the P2-P3 junction. Evidence that primary cleavage at sites other than 2A/2B and 2C/P3 occurs (albeit with less frequency) comes from the detection of polypeptides P2-3AB and 2C-3AB in PV-infected HeLa cells (58). Indeed, some P2-P3 processing at the 2C-3A cleavage site must occur, since polypeptides P2 and P3 are formed. However, we demonstrated here that P2 processing is very inefficient, which strongly suggests that the P2-P3 cleavage has no biological significance for the generation of P2-derived proteins. This alternative cleavage may represent a way of regulating the amount of P2- versus P3-derived proteins required for replication. The close relationship between processing of P2 and P3 proteins that we described in this report may have biological significance, since all (or nearly all) the P2- and P3-derived proteins participate in genome replication. It is possible that the synthesis of P2-derived proteins, in close association with the generation of P3-derived proteins, represents a very efficient way to assemble the protein component of the viral replication complexes required for genome amplification.

Finally, complementation analysis was performed to determine whether the RNA synthesis defect of 3AB mutants could be rescued by providing wild-type proteins in *trans*. None of the nonviable mutations or the temperature-sensitive mutant, 3AB-310/4, could be efficiently complemented by a PV-coxsackievirus recombinant virus (Fig. 9 and 10). This result is in contrast to a previous report by Bernstein and Baltimore (4), which described a 3A cold-sensitive mutant (mutant 3A-2) that could be complemented in *trans*. Mutant 3A-2 contained a Ser insertion between Thr-14 and Ser-15 within the sequence of 3A. Although this mutation affected vRNA synthesis, the defect was not strand specific, as for our mutant 3AB-310/4. In addition, mutant 3A-2 was defective only during the early (exponential) phase of RNA synthesis. The different phenotypes exhibited by mutants 3A-2 and 3AB-310/4 suggest that polypeptide 3AB participates in different functions required for RNA synthesis, some of which are complementable and some are not.

Different possible explanations may account for the lack of complementability of the mutations we created in the hydrophobic domain of 3AB. (i) The function provided by this domain (within 3AB) is *cis* acting, so a wild-type protein provided in *trans* would not be able to interact with and replicate the mutant template. (ii) The RNA containing 3AB mutations cannot function as a template for minus-strand RNA synthesis because the lesions we engineered caused perturbations of the RNA structure. (iii) Wild-type 3AB proteins are not able to reach the mutant template. Since 3AB is a membrane-associated protein (53, 58, 59) and vRNA synthesis occurs in a membranous environment (6, 7, 9–11, 20, 38, 48), it is likely that diffusion of proteins and RNA templated within the infected cell is very limited. Perhaps RNA templates and the proteins synthesized from them are sequestered within micro-membrane environments, creating limited-diffusion replication complexes so that the probability of interchange between proteins synthesized from different template is very low. (iv) The specific protein-protein interactions required for PV RNA synthesis actually take place immediately after the vRNA is translated and the viral polyprotein is synthesized. Once 2A is synthesized and P1 is cleaved from the polyprotein, the newly translated P2 and P3 precursors probably fold in a specific way to serve as a substrate for 3C, as well as to form (once they are cleaved) the specific protein-protein interactions needed for RNA synthesis, in particular for initiation of vRNA synthesis. Our finding that P3 mutations affected P2 processing suggests a strong conformational interaction between these two precursors. It is likely that once these viral protein complexes are formed, it is not possible (or it is very inefficient) to unfold and dissociate them to permit the exchange of a mutated protein by a functional wild-type protein. Thus, the mutated 3AB proteins would remain sequestered by other proteins in these complexes, and the wild-type 3AB proteins provided in *trans* would not be able to displace them, resulting in a lack of 3AB complementation. It has also been reported that functions provided by other P2- and P3-derived viral proteins, such as 2B and 3D (5, 25), are not able to be complemented in *trans*. The sequestering of PV nonstructural proteins in specific, limited-diffusion complexes may provide a mechanism for effectively increasing the local concentrations of polypeptides crucial to initiation of RNA synthesis, particularly at early times after infection when the absolute concentration of such proteins in the cell is very low.

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