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Role of a Viral Membrane Polypeptide in Strand-Specific Initiation of Poliovirus RNA Synthesis

CRISTINA GIACHETTI AND BERT L. SEMLER*

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717

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A molecular genetic analysis has been combined with an in vitro biochemical approach to define the functional interactions required for nucleotidyl protein formation during poliovirus RNA synthesis. A site-directed lesion into the hydrophobic domain of a viral membrane protein produced a mutant virus that is defective in RNA synthesis at 39°C. The phenotypic expression of this lesion affects initiation of RNA synthesis, in vitro uridylylation of the genome-linked protein (VPg), and the in vivo synthesis of plus-strand viral RNAs. Our results support a model that employs a viral membrane protein as carrier for VPg in the initiation of plus-strand RNA synthesis. Our data also suggest that a separate mechanism could be used in the initiation of minus-strand RNA synthesis, thereby providing a means for strand-specific regulation of picornavirus RNA replication.

Replication of the genomic RNAs of positive-strand viruses of the family *Picornaviridae* produces viral RNAs containing a small protein (VPg) covalently attached to their 5′ ends (11, 26). Specific viral polypeptides are required for the enzymatic activities associated with picornavirus RNA replication. For example, a primer- and RNA-dependent RNA polymerase (polypeptide $3D^{pol}$) is responsible for elongation of RNA strands (9, 10, 22, 37), and the covalent attachment of VPg to viral RNA is an absolute requirement for genome replication in vivo (28). However, the roles of other viral proteins and the participation of host cell proteins in RNA replication have not been clearly established (for reviews, see references 19, 29, and 31). The mechanism of initiation of viral RNA synthesis has proven to be especially intractable. For poliovirus, a member of the picornavirus group, two possible mechanisms for initiation of nascent RNA chains have been proposed. One model, based primarily on in vitro synthesis of minus-strand RNAs, involves a template priming step that requires a "host factor" (7) to act as a terminal uridylyltransferase and add a small number of uridylate residues to the 3′-terminal poly(A) of virion plus-strand RNA (2). A poly(A)-oligo(U) hairpin would then form and act as a primer for the elongation activity of $3D^{pol}$. The generation of unit-length product RNA linked to VPg is the result of a second step in which VPg (or a precursor of VPg) participates in a *trans*-esterification reaction to initiate a concerted cleavage and linkage step (35).

A second mechanism for initiation of poliovirus RNA synthesis has been proposed in which either VPg, a polypeptide precursor to VPg, or a uridylylated derivative of VPg acts as a primer for the viral RNA polymerase (25, 32, 33). The in vitro synthesis of VPgpU(pU) (the 5′-terminal moiety of both plus- and minus-strand RNAs) was demonstrated in a membranous replication complex (called the crude replication complex [CRC]) isolated from infected HeLa cells (33). It was later demonstrated that preformed VPgpU can be chased into VPgpUpU and nucleotidyl proteins containing nine or more of the poliovirus 5′-proximal nucleotides (32). The synthesis of VPgpU(pU) (as in the case of full-length viral RNA) is strictly dependent on the presence of intact membranes (32). These studies have also provided evidence for the role of cytoplasmic membranes in the initiation of RNA synthesis, since poliovirus RNA replication occurs on smooth membranes in infected HeLa cells (5). In addition, the following observations support a model in which VPgpU can function as a primer for poliovirus plus-strand synthesis within a membranous environment: (i) VPg is attached to the 5′ end of the RNA genome of poliovirus, to the 5′ end of the intracellular plus-strand RNA (except mRNA associated with polyribosomes) and minus-strand RNA, and to all nascent RNAs (26, 27); (ii) antibodies against VPg immunoprecipitate VPg as well as VPgpUpU from infected cells (6); and (iii) in in vitro replication reactions, anti-VPg antibody specifically inhibits initiation of viral RNA synthesis (3, 24).

Since VPg is a strongly polar (basic) protein, it must be delivered to the hydrophobic site by a lipophilic carrier. Three polypeptide precursors of VPg, P2-3AB, 2C-3AB, and 3AB, were found specifically associated with membrane fractions of poliovirus-infected cells, presumably because they contain a nonpolar sequence of 22 amino acids just amino terminal to the amino acid sequence of VPg (30, 34). The smallest VPg-containing polypeptide, 3AB (12,000 Da), is a likely candidate for a donor of VPg to the membrane-associated poliovirus RNA-synthesizing complex because it is an abundant viral product of proteolytic processing that appears to turn over very slowly (34). Moreover, inspection of the amino acid sequences of polypeptides 3AB of the three strains of poliovirus and of other closely related picornaviruses, coxsackievirus and human rhinovirus, indicates the presence of a similar, nonpolar amino acid sequence preceding VPg (12). Conservation of such a hydrophobic domain among different picornaviruses adds further support for its proposed role in viral RNA replication.

The existence of poliovirus cDNA clones which produce infectious virus upon transfection of primate cells makes it possible to genetically manipulate poliovirus RNA. We have used this approach to introduce amino acid substitutions into the hydrophobic domain of 3AB. Among the mutants that we isolated, mutant Se1-3AB-310/4 (containing a Thr-67-to-Ile

* Corresponding author.

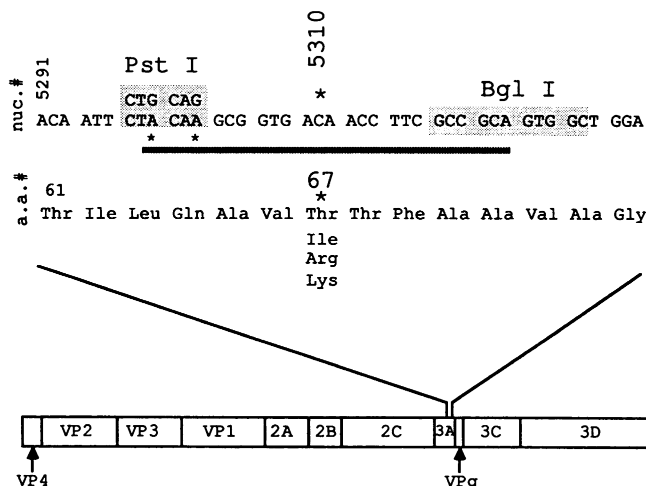

FIG. 1. Partial nucleotide (nuc.) and amino acid (a.a.) sequences of the hydrophobic domain of 3AB, showing the mutations that we engineered and the resulting change at amino acid 67. The asterisks at nucleotides 5299 and 5302 indicate the changes made by site-directed mutagenesis to introduce a new *Pst*I restriction site. ▮▮▮▮▮▮▮, Synthetic double-stranded oligonucleotide used to introduce the randomized nucleotide at position 5310.

change; hereafter referred to as 3AB-310/4) showed a highly temperature-sensitive phenotype (12). In this report, we show that the mutation affects initiation of RNA synthesis, in vitro uridylylation of VPg, and in vivo plus-strand RNA synthesis. These results provide evidence for a model in which 3AB serves as carrier for VPg to initiate plus-strand RNA synthesis. They also provide a possible mechanism for the differential control of viral RNA synthesis by strand-specific initiation mechanisms.

MATERIALS AND METHODS

Cells and virus stocks. HeLa cell monolayers were used throughout. The wild-type virus stock used for all experiments reported here was derived from a single-plaque isolate produced after transfection of HeLa cells with a cDNA clone of PV1 (M) RNA (plasmid pPVA55; 20), containing a new *Pst*I restriction site at N-5297. The viral growth and RNA and protein synthesis kinetics at 33 and 39°C exhibited by this virus are indistinguishable from those of the original wild-type virus.

Generation and isolation of amino acid replacement mutants. The generation, isolation, and partial characterization of poliovirus mutant 3AB-310/4 have been previously described (12). Briefly, we created amino acid replacement mutations in the hydrophobic domain of polypeptide 3AB by using a synthetic mutagenesis cassette. We first introduced a new *Pst*I site at position 5297 (21 nucleotides upstream of the *Bgl*I site at position 5318), using oligonucleotide-directed, site-specific mutagenesis (16) of a subgenomic poliovirus cDNA (Fig. 1). A synthetic pair of oligonucleotides, corresponding to the region of the poliovirus genome between N-5297 and N-5323 and containing nucleotides randomized at position 5310, was inserted between the *Pst*I and *Bgl*I restriction sites. Putative mutants were screened by sequencing of double-stranded plasmid DNA, and a *Bgl*II (N-5601)-to-*Bam*HI (N-4600) fragment containing the specific changed sequence was used to replace the corresponding fragment of a full-length infectious plasmid, pPVA55 (20).

Mutant plasmids were transfected into HeLa cell monolayers at different temperatures by the calcium phosphate coprecipitation technique (14). Dilutions of the supernatant from the mutant cDNA 310/4 transfection at 33°C were used to infect cells under semisolid agar. One well-isolated plaque was used to generate a virus stock (passage 2). RNA was extracted from passage 2 mutant virus 3AB-310/4, and the presence of the introduced mutation was confirmed by dideoxynucleotide sequence analysis using reverse transcriptase.

Labeling of infected cells with [^{3}H]uridine. HeLa cell monolayers in 24-well dishes (approximately 10^5 cells per well) were infected with wild-type or mutant virus stocks at a multiplicity of infection of 50. After a 30-min adsorption period, Dulbecco modified Eagle medium supplemented with 5% fetal calf serum was added, and cells were incubated at 33 or 39°C. At the indicated times, dactinomycin (5 μg/ml, final concentration) and [^{3}H]uridine (5 μCi/ml, final concentration) were added. At different times postinfection, the medium was discarded and the cells were resuspended in 1 ml of phosphate-buffered saline. The resuspended cells were treated with trichloroacetic acid (TCA), and the precipitates were collected on glass fiber filters. Radioactivity on the filters was determined in a liquid scintillation counter.

Determination of in vivo RNA synthesis by Northern (RNA) blot analysis. HeLa cell monolayers in 60-mm petri dishes were infected with wild-type or mutant virus stocks at a multiplicity of infection of 50. At various times after infection, total intracellular RNA was extracted as previously described (8). Total cytoplasmic RNA (10 μg) from each sample was denatured with glyoxal and dimethyl sulfoxide. After denaturation, each sample was divided into two equal portions, and each portion was fractionated (in parallel) in two 1% agarose-NaH_2PO_4 gels as described by Maniatis et al. (23). RNA contained in each gel was transferred (in parallel) to two GeneScreen Plus membranes (Dupont). The efficiency of the transfer was analyzed by staining the gels with ethidium bromide after the transfer. Membranes were prehybridized for approximately 4 h at 50°C in 5× Denhardt solution–6× SSC (0.9 M sodium chloride, 0.09 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS)–500 μg of denatured salmon sperm DNA per ml. Hybridization was for approximately 18 h at 50°C in 1× Denhardt solution–5× SSC–0.5% SDS–500 μg of denatured salmon sperm DNA per ml and ^{32}P-labeled poliovirus-specific synthetic oligonucleotide probes. After hybridization, membranes were washed in 6× SSC–0.5% SDS three times for 10 min at room temperature and one time at 50°C for 30 min and then one time in 2× SSC–2% SDS at 55°C for 30 min. Hybridized filters were exposed to XAR film for different amounts of time.

Synthesis of VPgpU(pU) and RNA in vitro. CRCs were prepared from mutant- or wild-type-infected HeLa cells as described by Takeda et al. (32), with the modifications described below. Wild-type-infected cells were incubated at 37°C and harvested at 5 h postinfection. Mutant-infected cells were incubated at 33°C and harvested at 12 h postinfection. In each case, 8×10^6 cells were infected. CRCs were resuspended in 80 μl of buffer (10 mM Tris-HCl [pH 8], 10 mM NaCl, 15% glycerol), divided into aliquots, and stored at −70°C.

In vitro syntheses of VPgpU(pU) and RNA were carried out by incubation of 2-μl aliquots of CRC in 20 μl of the standard reaction mixture described by Takeda et al. (32). At the indicated times, 25 μCi of [α-^{32}P]UTP was added to the

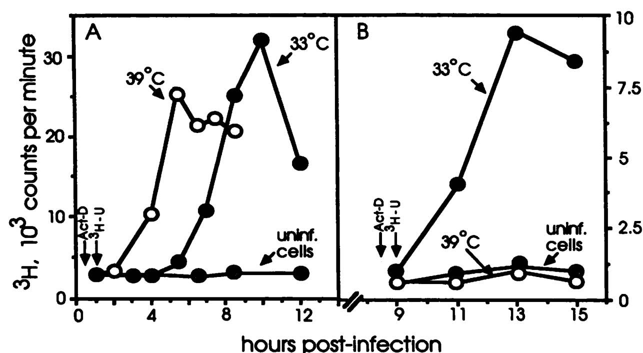

FIG. 2. Kinetics of viral RNA synthesis induced in HeLa cells by wild-type (A) and mutant 3AB-310/4 (B) virus. HeLa cells infected with wild-type or mutant virus were incubated at 33 or 39°C. Dactinomycin (Act-D; final concentration, 5 μg/ml) and [^{3}H] uridine (^{3}H-U; final concentration, 5 μCi/ml) were added at the times indicated. At different times postinfection, aliquots were withdrawn and incorporation of [^{3}H]uridine into TCA-precipitable material was determined. uninf., Uninfected.

in vitro reaction mixtures. After different times of incubation at 33 or 39°C, the reactions were stopped by addition of SDS to a final concentration of 0.5%. One-fifth of each reaction mixture was used to analyze RNA synthesis, and the rest was used to detect synthesis of VPgpU(pU). To detect viral RNA synthesis, the reaction mixture was treated with proteinase K in the presence of 2% Sarkosyl, and RNA was extracted with phenol-$CHCl_3$ (1:1), precipitated with ethanol, and analyzed by 0.8% agarose gel electrophoresis. Analysis of VPgpU(pU) synthesis was carried out by immunoprecipitation of ^{32}P-labeled VPgpU(pU) with a 1:1 mixture of anti-VPg (C7) (30) and anti-VPg (N10) (33). Immunoprecipitations were carried out as described by Takeda et al. (32). Immunoprecipitated VPgpU(pU) was then subjected to electrophoresis on a 20% SDS-polyacrylamide minigel (Bio-Rad), using a Laemmli gel buffer system with a separating gel of pH 8 instead of the normal pH 8.8 separating gel.

RESULTS

Viral RNA synthesis in mutant-infected cells at permissive and nonpermissive temperatures. The initial characterization of mutant 3AB-310/4 indicated that the change we introduced in the hydrophobic domain of polypeptide 3AB (Thr-67 to Ile; Fig. 1) primarily affected viral RNA synthesis (12). To further analyze the characteristics of the viral RNA synthesis induced by mutant 3AB-310/4 at the permissive (33°C) and nonpermissive (39°C) temperatures, we first determined the kinetics of incorporation of [^{3}H]uridine into TCA-insoluble material of mutant- and wild-type virus-infected cells in the presence of dactinomycin. Mutant 3AB-310/4 exhibited a delayed kinetics of viral RNA synthesis (Fig. 2B), and the maximum level of RNA synthesized was about three times lower than the maximum level of RNA synthesized by the wild-type virus at the permissive temperature (Fig. 2A). At 39°C, the mutant induced extremely low levels of viral RNA synthesis: the amount of [^{3}H]uridine-labeled TCA-precipitable material detected at different times postinfection was similar to the amount detected in uninfected cells. Since the stability (at 39 or 33°C) of the RNA synthesized by the mutant at the permissive temperature was similar to that of wild-type RNA (not shown), we concluded that the lesion we introduced in polypeptide 3AB affected the viral RNA synthetic process.

To determine whether the 3AB mutation differentially affected plus- or minus-strand viral RNA synthesis, we used Northern blots to analyze the amount of plus- and minus-strand RNAs present in wild-type- or mutant-infected cells at different times postinfection. In three independent experiments (of which Fig. 3 is an example), we found that the maximum level of plus-strand RNA synthesized by the mutant at the permissive temperature was about 1.5 to 3 times lower than the amount synthesized by the wild-type virus at 33 or 39°C. Minus-strand RNA synthesis was normal or reduced up to twofold with respect to the values obtained for the wild-type virus. At the nonpermissive temperature, the mutant induced almost undetectable levels of plus- or minus-strand RNA synthesis, and very long exposures of the Northern blots were required to detect the presence of viral RNA. Laser densitometric quantitation of appropriate exposures of the autoradiograms showed that plus-strand RNA synthesis induced by the mutant at 39°C was reduced by 250- to 400-fold compared with the wild-type synthesis, while minus-strand synthesis was reduced only by about 10- to 25-fold (Table 1). Therefore, the minus-strand RNA/plus-strand RNA synthesis ratio induced by the mutant at 39°C was at least 10 times higher than the ratio induced by wild-type virus. This result indicated that the mutation in polypeptide 3AB affected mostly plus-strand RNA synthesis.

We next carried out temperature shift experiments to further analyze the RNA synthesis defect of 3AB-310/4. In the experiment shown in Fig. 4, mutant-infected cells were

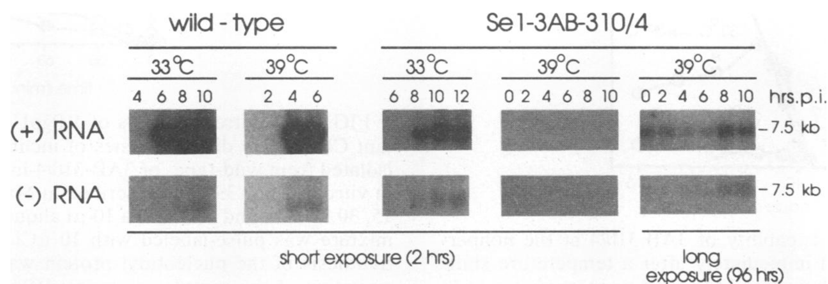

FIG. 3. Plus-strand [(+)] and minus-strand [(−)] viral RNA synthesized in wild-type- or mutant-infected cells at permissive and nonpermissive temperatures. HeLa cell monolayers infected with wild-type or mutant virus were incubated at 33 or 39°C. At different times after infection, total intracellular RNA was extracted, denatured with glyoxal, separated in agarose gels, and transferred to GeneScreen Plus membranes (Dupont). Membranes were hybridized to ^{32}P-labeled poliovirus-specific probes designed to detect plus- or minus-strand viral RNA. Only the portions of the autoradiograms in which full-length viral RNA was detected are shown.

TABLE 1. Quantitation of plus- and minus-strand viral RNA synthesis induced by wild-type or mutant poliovirus at 33 and 39°C[a]

RNA	Wild type				3AB-310/4			
	33°C		39°C		33°C		39°C	
	8[b]	10	4	6	10	12	8	10
Plus strand	279	386	405	325	112	123	1.5	0.99
Minus strand	8.25	13.5	12.5	13.5	12.5	15.2	0.5	0.45
Ratio, plus/minus strand	33.8	28.6	32.4	25	8.9	8	3	2.2

[a] Appropriate exposures of the autoradiograms shown in Fig. 3 were quantitated by using a laser densitometer LKB UltroScan XL. Numbers shown are arbitrary units reflecting the relative density of the autoradiographic image produced following exposure of the Northern blots to XAR film.
[b] Hours postinfection.

incubated at the permissive temperature for 9.5 h. At this time, half of the infected cells were shifted to the nonpermissive temperature, and the other half of the culture was maintained at 33°C. Dactinomycin and [^{3}H]uridine were added 30 min before the shift and at the time of the shift, respectively. Incorporation of [^{3}H]uridine into TCA-precipitable material was determined at different times after the shift. Mutant 3AB-310/4 was able to synthesize RNA at a normal rate immediately after the shift, but longer incubation at 39°C (2 h or more) resulted in loss of the RNA synthetic capability at 39°C. This result suggested that the replicative complexes that had been assembled at 33°C were functional at 39°C. The loss of RNA synthesis activity 2 h after the shift-up was consistent with a defect in the initiation of RNA synthesis or in the assembly of replication complexes at the nonpermissive temperature as being responsible for the temperature-sensitive RNA synthesis phenotype of mutant 3AB-310/4.

In vitro RNA synthesis analysis of mutant 3AB-310/4. Since the in vivo analysis of mutant 3AB-310/4 suggested a defect in initiation of RNA synthesis, we decided to study the synthesis of nucleotidyl protein [VPgpU(pU), the proposed primer for the polymerase (25, 32, 33)] induced by CRC isolated from mutant-infected HeLa cells. We first compared the synthesis of nucleotidyl protein induced by mutant and wild-type CRCs after different times of in vitro incubation at the permissive or nonpermissive temperature and calculated the ratios of VPgpU(pU) synthesis at 33 versus 39°C for mutant and wild-type extracts after different times of incubation. We observed (Fig. 5) that the formation of nucleotidyl protein by wild-type CRC was slightly less efficient at 39 than at 33°C. The ratio of synthesis of nucleotidyl protein (at 33 versus 39°C) calculated for mutant CRC was always higher than the ratio obtained for wild-type CRC. This difference increased sharply after 60 min of incubation, so that after 2 h of incubation the ratio obtained for the mutant was approximately eight times higher than the ratio obtained for the wild type. This result suggested that the formation of nucleotidyl protein in mutant 3AB-310/4 was more temperature sensitive than that of the wild-type virus and that the defect required ~30 to 60 min of incubation at the nonpermissive temperature before it was clearly expressed.

We next analyzed the kinetics of synthesis of nucleotidyl protein in crude membrane extracts isolated from mutant-infected cells that were shifted to the nonpermissive temperature and incubated at that temperature for 2 h before harvesting. These results were compared with those obtained with wild-type extracts and with mutant extracts isolated from cells incubated at 33°C throughout the infection. In the same experiment, we also analyzed the kinetics of RNA synthesis induced by these three different extracts. The kinetics of synthesis of VPgpU(pU) in wild-type CRC were similar at both temperatures (Fig. 6A). There was a linear increase in the amount of nucleotidyl protein synthe-

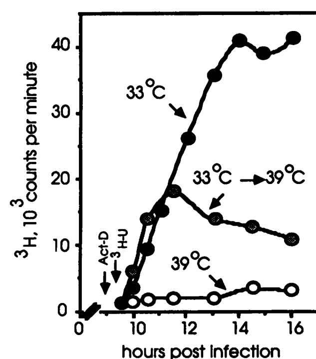

FIG. 4. RNA synthesis capability of 3AB-310/4 at the nonpermissive temperature (39°C) immediately after a temperature shift-up. HeLa cells infected with mutant 3AB-310/4 were incubated at 33 or 39°C. At 9.5 h postinfection, part of the infected cultures incubated at 33°C were shifted to 39°C. Dactinomycin (Act-D) and [^{3}H]uridine (^{3}H-U) were added at the times indicated. Incorporation of [^{3}H]uridine into TCA-precipitable material was determined at different times postshift.

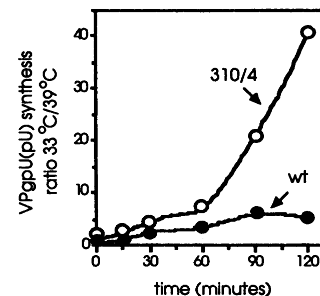

FIG. 5. In vitro synthesis of VPgpU(pU) by wild-type and mutant CRCs after different times of incubation at 33 or 39°C. CRCs isolated from wild-type- or 3AB-310/4-infected cells were incubated in vitro at 33 or 39°C as described in Materials and Methods. At 0, 15, 30, 60, 90, and 120 min, a 10-μl aliquot from the in vitro reaction mixture was pulse-labeled with 10 μCi of [α-^{32}P]UTP for 15 min. Synthesis of the nucleotidyl protein was detected by immunoprecipitation of the samples with anti-VPg antibody, and the precipitates were separated by electrophoresis on 20% polyacrylamide gels. The amount of nucleotidyl protein synthesized in each case was determined by laser densitometric scanning of appropriate exposures of the autoradiograms, and the ratios of synthesis at 33 versus 39°C for wild-type (wt) or mutant reactions were determined.

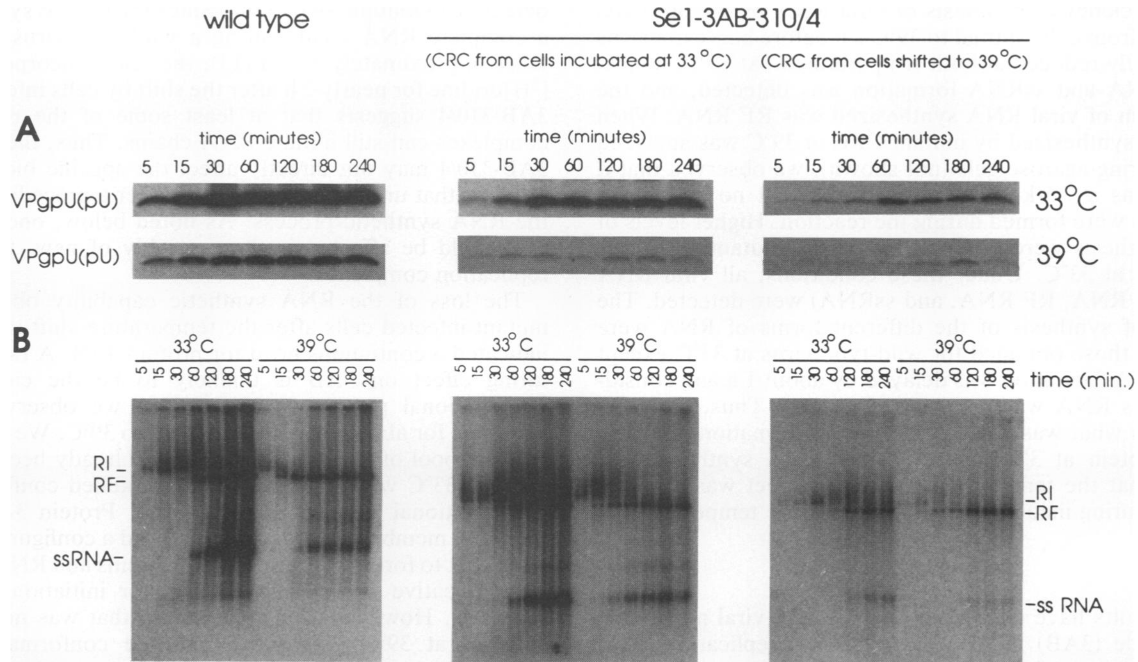

FIG. 6. Kinetics of synthesis of RNA and nucleotidyl protein in crude membrane fractions isolated from wild-type- or mutant-infected cells incubated at 33°C or shifted for 2 h at 39°C before harvesting. Crude membrane complexes isolated from wild-type- or mutant 3AB-310/4-infected cells were incubated at 33 or 39°C as described for Fig. 5. After 15 min of preincubation at the indicated temperatures, [α-^{32}P]UTP was added. At 5, 15, 30, 60, 120, 180, and 240 min, 10-μl aliquots were withdrawn and the synthesis of nucleotidyl protein (A) and in vitro-synthesized RNA products (B) was determined. Synthesis of VPgpU(pU) at different times was determined as described for Fig. 5. Only the lower portions of the autoradiograms, in which the nucleotidyl proteins were detected, are shown. The in vitro-synthesized RNA products were recovered by extraction with phenol-choroform (1:1) and analyzed by 0.8% nondenaturing agarose gel electrophoresis. Shown are the autoradiograms of the gels. The gels containing mutant samples were exposed four times longer than those containing wild-type samples.

sized during the first 30 min of incubation, and after this time the amount of VPgpU(pU) detected remained almost constant. Laser densitometric scanning of appropriate exposures of the autoradiograms indicated that the maximum level of VPgpU(pU) present in wild-type in vitro reaction mixtures incubated at 39°C was about two times lower than the maximum level reached at 33°C. The kinetics of synthesis at the permissive temperature of VPgpU(pU) by CRC isolated from mutant-infected cells incubated at 33°C were somewhat similar to the ones showed by wild-type CRC, but the maximum amount of nucleotidyl protein synthesized was about two times lower than that synthesized in wild-type reaction mixtures incubated at 33°C. At the nonpermissive temperature, the amount of nucleotidyl protein synthesized by mutant CRC was considerably reduced. After 15 min of incubation, the maximum level was reached. This level of synthesis was approximately 7 times lower than the level achieved by the mutant CRC at 33°C, producing a 39-to-33°C ratio 3.5 times lower than that obtained for the wild-type virus. These results provide independent confirmation of our previous result (Fig. 5). Taken together, our data indicate that synthesis of VPgpU(pU) by mutant 3AB-310/4 is thermosensitive. Moreover, CRC prepared from mutant-infected cells that had been shifted to 39°C for 2 h before harvesting resulted in extremely reduced synthesis of VPgpU(pU) at 39°C. Interestingly, when this mutant CRC was incubated at 33°C, a clear increase in nucleotidyl protein synthesis was observed during the first hour of incubation, indicating that the temperature-sensitive defect was partially reversed.

Analysis of the kinetics of in vitro synthesis of the different forms of viral RNA induced by wild-type CRC showed that all three virus-specific RNA species (replicative intermediate [RI], replicative form [RF], and single stranded) were synthesized at different rates at both temperatures (Fig. 6B). As expected, RI RNA synthesis was detected at earlier times of incubation than were the other forms of viral RNA. At 39°C, formation of RI RNA diminished rapidly, so that after 30 min of incubation, RF RNA and single-stranded RNA (ssRNA) were the only two viral RNA forms detected. These forms reached their maximum levels of synthesis by 2 h of incubation. At 33°C, synthesis of RI RNA continued for a longer period of time. RI synthesis reached a maximum level after 1 h of incubation, and this level remained almost constant during the rest of the assay. Wild-type ssRNA was detected after 1 h of incubation. Note that increasing levels of synthesis of ssRNA were observed up to 4 h of incubation at 33 but not 39°C, resulting in formation of higher overall levels of ssRNA at 33°C. A similar result was observed when we analyzed viral RNA synthesized in vitro, at 33 or 39°C, by mutant CRC isolated from cells incubated at 33°C. All three virus-specific RNA species (RI RNA, RF RNA, and ssRNA) were synthesized by mutant CRC at both temperatures, in ratios similar to those observed for wild-type virus. The only difference observed was an overall reduction in the amount of all different species of RNA formed at both temperatures (note the different exposure times of the autoradiograms for wild-type and mutant extracts). This result indicated that the RNA synthetic process exhibited by mutant 3AB-310/4 in vitro is less efficient.

The efficiency of synthesis of viral RNA by mutant CRC prepared from cells shifted to 39°C 2 h before harvesting was dramatically reduced at both temperatures. At 39°C, almost no RI RNA and ssRNA formation was detected, and the major form of viral RNA synthesized was RF RNA. When the RNA synthesized by mutant CRC at 39°C was analyzed in denaturing agarose gels (not shown), we observed that it migrated as a 7.5-kb RNA, suggesting that no dimer-size molecules were formed during the reaction. Higher levels of RNA synthesis were observed when the mutant CRC was incubated at 33°C. Under these conditions, all viral RNA forms (RI RNA, RF RNA, and ssRNA) were detected. The kinetics of synthesis of the different forms of RNA were similar to those obtained for wild-type virus at 33°C except that ssRNA formation was delayed by about 1 h and considerably less RNA was synthesized overall. Thus, in agreement with what was detected above for formation of nucleotidyl protein at 33°C, our in vitro RNA synthesis data suggest that the temperature-sensitive defect was partially rescued during incubation at the permissive temperature.

DISCUSSION

Our results have addressed the role of a viral membrane polypeptide (3AB) in picornavirus RNA replication. Two unique features of the RNA synthetic process suggest the need for such a protein. First, the generation of VPg-linked RNA requires cleavage of VPg from a precursor polypeptide prior to or shortly after the formation of nucleotidyl protein complex. Second, the synthesis of viral RNAs on membrane-bound replication complexes may require a specific association with a hydrophobic stretch of amino acids in a viral replication protein, either as a means of physically linking the complex to membranes or as a means of delivery of the charged peptide (VPg) to the hydrophobic environment found in such complexes. The genetic and biochemical results with our mutant (3AB-310/4) provide evidence for the role of protein 3AB in this unique replication process. It is of interest that a poliovirus mutant in protein 3A was isolated by Bernstein and Baltimore (4). The mutant, 3A-2, contained an amino acid insertion near the amino terminus of protein 3A. This mutation produced a cold-sensitive virus defective in plus- and minus-strand viral RNA synthesis at the nonpermissive temperature. Temperature shift experiments suggested that the altered function was expressed only during the early (exponential) phase of RNA synthesis. The in vivo and in vitro analyses of 3AB-310/4 reported here indicate that the phenotypic expression of 3A-2 is very different from that of mutant 3AB-310/4. It appears that polypeptide 3AB may be composed of different domains that affect different functions in RNA synthesis.

The temperature shift experiment (described above) indicated that we were not able to completely rescue the RNA-minus phenotype of mutant 3AB-310/4. RNA synthesis was normal immediately after the shift, indicating that the elongation function was not affected by the mutation (Fig. 4). This was expected, since $3D^{pol}$ is the only viral protein required for elongation (9, 10, 22, 37). The normal kinetics of viral RNA synthesis induced by CRC isolated from HeLa cells infected with 3AB-310/4 at 33°C but incubated in vitro at 39°C confirmed this result (Fig. 6B). In addition, the synthesis of ssRNA observed in such extracts incubated at 39°C indicated that the release of newly synthesized RNA strands from the replicative complexes was not defective (Fig. 6B). These results strongly suggest that one of the functions required for initiation of viral RNA synthesis was defective in mutant 3AB-310/4. Since the in vivo synthesis of a complete RNA chain during a wild-type virus infection takes approximately 1 min (13), the linear incorporation of [^{3}H]uridine for nearly 2 h after the shift by cells infected with 3AB-310/4 suggests that at least some of the replication complexes can still initiate RNA chains. Thus, the lesion in 3AB-310/4 may not directly affect the specific biochemical reaction that uridylylates VPg but rather a preceding step in the RNA synthetic process. As noted below, one function that could be affected is the assembly of new, functional replication complexes.

The loss of the RNA synthetic capability observed in mutant-infected cells after the temperature shift-up (Fig. 4) indicated a continuous need for protein 3AB. A rapid denaturing effect on 3AB is unlikely to be the cause of a nonfunctional protein at 39°C, since we observed RNA synthesis for almost 2 h after the shift to 39°C. We speculate that the pool of 3AB protein that had already been synthesized at 33°C was (although with a modified conformation) still functional after the shift to 39°C. Protein 3AB maintained its membrane association (34) and a configuration that allowed it to form (with other viral proteins and RNA) part of the replicative complexes required for initiation of RNA synthesis. However, the 3AB protein that was newly synthesized at 39°C assumed an altered conformation that affected correct insertion in the membrane and association with other viral proteins. This resulted in formation of replication complexes that were severely impaired for initiation of RNA synthesis at 39°C. The mutation in 3AB probably results in an inefficient delivery of VPg, so the availability of VPg for the uridylylation reaction will be limited. The lag time that we observed before full expression of the RNA synthetic defect by mutant 3AB-310/4 after the shift-up could represent the time necessary to recycle new (i.e., defective) replication complexes to the membrane, during which time the pool of VPg is slowly being depleted.

The mutation that we introduced in 3AB-310/4 produces a change of Thr to Ile, which should increase the hydrophobicity of 3AB. As we would predict, changes in the absolute association of 3AB or 3A polypeptides with membranes of the infected cells were not seen (data not shown). Thus, a correlation between deficient RNA synthesis and association of 3AB with membranes could not to be established for this mutation. However, the other two mutations that we created by using the mutagenesis cassette that was used to generate 3AB-310/4 (Fig. 1) produce changes of Thr to Arg or to Lys, two positively charged amino acids. Plasmids containing these two different lesions were nonviable in cDNA transfection experiments, suggesting that the hydrophobic domain in 3AB is essential. In addition, viral RNAs derived from such plasmids by in vitro transcription are unable to replicate following transfection of HeLa cells (12a).

Synthesis of VPgpU(pU), a proposed primer for viral RNA synthesis, induced by mutant extracts incubated at 39°C was less efficient than the synthesis induced by wild-type extracts at the same temperature (Fig. 5). The in vitro kinetics of synthesis of nucleotidyl protein and their correlation with the kinetics of synthesis of different viral RNA forms (Fig. 6) strongly suggest that the two processes are related. For the CRC isolated from mutant-infected cells that had been shifted to 39°C 2 h before harvesting, the loss of VPgpU(pU) synthesis at 39°C coincided well with almost no RI RNA and ssRNA synthesis at 39°C. This in vitro result correlates well with the in vivo temperature-sensitive RNA synthesis defect of the mutant. Apparently, a longer preincubation of mutant infected cells at 39°C before preparation

of the CRC resulted in a reduced ability to uridylylate VPg and to synthesize RNA in vitro. This suggests that continuous incubation of mutant infected cells at 39°C may lead to a large amplification of the defect that we observed in vitro, resulting in the very dramatic reduction of mutant RNA synthesis found in vivo at 39°C. When the same extract was incubated in vitro at 33°C, a slow recovery of synthesis of nucleotidyl protein was observed that correlates well with increasing levels of RI RNA and ssRNA synthesis at this temperature. These results are consistent with a model in which VPgpU(pU) acts as primer for the viral polymerase and 3AB serves as carrier for VPg, thus participating in initiation of viral RNA synthesis.

It is of interest that temperature-sensitive synthesis of nucleotidyl protein was reported for the attenuated Sabin derivative of poliovirus type 1 (36). A molecular genetic analysis demonstrated that the defect was associated with the viral polymerase, suggesting that uridylylation of VPg may be catalyzed by $3D^{pol}$. Although extensive mutational analysis of VPg has been described, no temperature-sensitive mutants mapping in VPg have been isolated to date (17, 18, 28).

The mutation that we engineered in 3AB-310/4 affected the kinetics of viral RNA synthesis at permissive and nonpermissive temperatures (Fig. 2). At 33°C, RNA synthesis was less efficient, so delayed kinetics and lower yields of RNA were observed. At 39°C, almost no RNA synthesis was detected. This in vivo analysis correlated well with the less efficient in vitro kinetics of viral RNA synthesis detected in the mutant CRC (Fig. 6B). Interestingly, plus-strand RNA synthesis was dramatically reduced during infection of HeLa cells by 3AB-310/4 at 39°C (Fig. 3), while minus-strand synthesis was reduced to a lesser extent, probably as a consequence of the reduced number of plus-strand RNA templates. In addition, mutations that disrupt the formation of a ribonucleoprotein complex at the 5′ end of poliovirus RNA that selectively affected positive-strand RNA accumulation have been described recently (1). This complex may play a role in organizing viral and cellular proteins involved in plus-strand RNA synthesis. Since the viral RNA polymerase is able to elongate both strands, the differences in plus- and minus-strand RNA yields in mutant-infected cells raise the possibility that two different mechanisms for initiation of poliovirus RNA synthesis are involved.

As a result of our data demonstrating that mutant 3AB-310/4 is impaired in plus-strand RNA synthesis, we suggest that the protein-nucleotidyl protein primer model for initiation of RNA synthesis may, in part, provide a mechanism for generation of plus strands during poliovirus infection. Such a model accounts for RNA synthesis taking place in a membranous environment and provides a mechanism for the delivery of a hydrophilic protein (VPg) to RNA chains via a lipophilic carrier polypeptide (3AB). We have shown here that a lesion in 3AB impairs both the production of uridylylated VPg and in vivo initiation of plus-strand RNA synthesis, providing biochemical and genetic evidence for the mechanism described above. Our results do not preclude a separate model that has been proposed to explain the in vitro generation of minus-strand RNAs. Such a model accounts for the observed in vitro synthesis of dimer-size RNA molecules by the activity of purified 3D RNA polymerase and a relatively crude preparation of host factor (15, 21, 38). Such dimers have been recently shown to be templates for a self-catalyzed VPg linkage reaction that produces unit-length minus strands attached to VPg via a tyrosine-phosphate bond (35). This latter activity in RNA synthesis should not be affected by the mutation that we engineered in 3AB-310/4. Indeed, we found that minus-strand RNA synthesis was not primarily affected in mutant-infected HeLa cells. We were also unable to detect an increased accumulation of dimer-length RNA molecules in cells infected with 3AB-310/4 at 39°C or in in vitro RNA synthesis reactions at 39°C using a CRC from mutant-infected cells. It will be important to determine whether such differential mechanisms for initiation of RNA synthesis can account for multiply initiated nascent plus strands in the form of RI RNA that uses a minus-strand template and single-run synthesis of minus strands in the form of RF molecules that use a plus-strand template.

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