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Critical Residues of Semliki Forest Virus RNA Capping Enzyme Involved in Methyltransferase and Guanylyltransferase-Like Activities

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The Semliki Forest virus (SFV) replicase protein nsP1 has methyltransferase (MT) and guanylyltransferase-like (GT) activities, which are involved in the capping of viral mRNAs. MT catalyzes the transfer of the methyl group from *S*-adenosylmethionine (AdoMet) to position 7 of GTP, and this reaction is followed by GT-catalyzed formation of the covalent complex m^7GMP-nsP1. These reactions are virus specific and thus potential targets for inhibitors of virus replication. We have mutated residues of SFV nsP1, which are conserved in related proteins of the large alphavirus-like superfamily. Mutations of D64, D90, R93, C135, C142, and Y249 to alanine destroyed or greatly reduced the MT activity of nsP1. All MT-negative mutants lost also the GT activity, confirming that methylation of GTP is an essential prerequisite for the synthesis of the covalent guanylate complex. Mutation of H38 prevented the GT reaction without destroying MT activity. Conservation of residues essential for both reactions in the alphavirus-like superfamily implies that they use a capping mechanism similar to that for the alphaviruses. Residues D64 and D90 were necessary for AdoMet binding, as measured by UV cross-linking. Secondary structure predictions of nsP1 and other proteins of the superfamily place these residues in positions corresponding to AdoMet-binding sites of cellular methyltransferases, suggesting that they all may be structurally related.

The methylated cap structure 7-methyl-G(5′)ppp(5′)NpNpNp... at the 5′ end of eukaryotic mRNAs is essential for efficient initiation of translation and mRNA stability and transport. The cap is usually synthesized in the nucleus by the sequential action of three enzymatic activities: mRNA triphosphatase, guanylyltransferase (GT), and finally, guanine-7-methyltransferase (MT) (reviewed in reference 18). Most RNA viruses replicating in the cytoplasm of eukaryotic cells must provide their own enzymes for the capping of their mRNAs. However, at least the picornavirus-like superfamily of RNA viruses has bypassed this requirement by including an internal ribosome entry site in their mRNAs (22). In this study we focus on the putative capping enzymes of the alphavirus-like superfamily of positive-strand RNA viruses. This superfamily includes alphaviruses, hepatitis E virus, and rubella virus, which are animal viruses, as well as a large number of plant viruses: tobra-, tobamo-, bromo-, tymo-, potex-, and carlaviruses and other groups (11). Their nonstructural (replicase) proteins have domains with limited sequence similarity to other polymerases (26) and helicases (6) and a third conserved domain, which is not found in other virus groups and has no identified cellular homologs (1, 29). Alphavirus nonstructural proteins are synthesized as a large polyprotein, which is autocatalytically cleaved into four proteins (nsP1 to nsP4). nsP4 contains the polymerase motifs, nsP3 is a phosphoprotein with unknown function, nsP2 contains the putative helicase domain, and nsP1 contains the third conserved domain (34).

Functional data concerning the third conserved domain are available only for nsP1 of two alphaviruses, Semliki Forest virus (SFV) and Sindbis virus. nsP1 catalyzes the MT reaction: *S*-adenosylmethionine (AdoMet) reacts with GTP to yield 7-methyl-GTP (m^7GTP) and *S*-adenosylhomocysteine (AdoHcy) (13, 31). The reaction is specific for GTP and dGTP, while the cap analog GpppA and capped RNA molecules are not methylated (13). In the presence of AdoMet, GTP also reacts with nsP1 to form a unique covalent complex, 7-methyl-GMP-nsP1 (2). This reaction resembles the action of cellular GTs and will be referred to as the GT reaction in this article. The MT reaction can take place in the presence of EDTA, while the GT reaction requires divalent cations (Mg^{2+} or Mn^{2+}). Other cellular and viral enzymes involved in mRNA capping form a covalent complex only with GMP as an intermediate in the GT reaction (18, 33, 35). The novel specificities of nsP1-catalyzed reactions have led us to propose that RNA capping of alphaviruses consists of the following reactions: GTP is first methylated and then forms the m^7GMP-nsP1 complex, from which 7-methyl-GMP is transferred to the mRNA to create the cap structure (2).

SFV nsP1 is also known to be palmitoylated and tightly associated with membranes, both in virus-infected cells and in cells expressing nsP1 in the absence of other virus-specific proteins (24). Furthermore, genetic experiments have demonstrated that Sindbis virus nsP1 is required for the synthesis of minus-strand RNA (30, 37).

In order to gain structure-function insights concerning the enzymatic functions of SFV nsP1, we have mutated amino acid residues, which are conserved in the alphavirus-like superfamily. Mutations in many of these residues affected the enzymatic activities, and mutants D64A and D90A were specifically defective in AdoMet binding. These results revealed conserved residues, which may be directly also involved in MT and GT activities in other proteins of this large viral superfamily.

MATERIALS AND METHODS

DNA constructs and their expression. Point mutations were constructed in plasmid pTSF1 (24), a derivative of pGEM3 (Promega), where the SFV nsP1 coding region is under the T7 promoter. The unique site elimination method (U.S.E. mutagenesis kit, Pharmacia Biotech) was applied according to the man-

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ufacturer's instructions. Point mutations generated usually caused a change of two nucleotides relative to the wild-type sequence. Two small deletions, constructs 1-515 and 1-478, were created similarly, by introducing two consecutive in-frame stop codons. Construct 1-429 was generated by PCR (14). Presence of desired mutations and absence of additional mutations was verified by automated DNA sequencing. The constructs in pTSF vector were used for transfection of HeLa cells infected with the recombinant vaccinia virus vTF7-3 producing the T7 polymerase (5), and the cells were labeled with [^{3}H]palmitate, as described previously (24).

For expression in *Escherichia coli*, the sequences coding for nsP1 mutants (*Nco*I-*Hin*dIII fragment) were transferred to plasmid pBAT4 (25) and expressed as described previously (13), except that the lysis buffer used contained 50 mM Tris-HCl (pH 8.0), 0.1% Tween 20, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 50 mM NaCl. The bacterial lysate was centrifuged for 20 min at 15,000 × *g*, and the resulting supernatant was used for further analysis.

Enzyme assays. Supernatant of the bacterial lysate was directly used for enzymatic and other experiments, as no method for purification of nsP1 has been found (histidine-tagged protein, denaturation-renaturation, and traditional methods have been extensively tested). As *E. coli* does not contain activities typical for nsP1, the background in these assays was below 0.5% of wild-type nsP1 activity. MT activity was assayed in a 25-μl final volume containing 100 mM HEPES (pH 8.0), 4 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM GTP, 10 μM AdoMet, and 0.75 μCi of *S*-adenosyl[methyl-^{3}H]methionine (87 Ci/mmol) for 30 min at 30°C. Labeled product was isolated in small DEAE-Sephadex columns and quantitated by liquid scintillation (13).

Covalent guanylate complex formation reaction mixtures were incubated in a 30-μl volume containing 5 μCi of [α-^{32}P]GTP (400 Ci/mmol) in 50 mM Tris-HCl (pH 7.5), 10 mM KCl, 2 mM $MgCl_2$, 5 mM dithiothreitol, and 100 μM AdoMet for 20 min at 30°C, as described previously (2). With [^{14}C]m^{7}GTP (used at 250 μM) the same conditions were present, except that 100 μM AdoMet was replaced by 100 μM AdoHcy.

Cross-linking studies. Cross-linking buffer contained 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, and 2 mM EDTA. In AdoMet cross-linking (8) 25-μl reaction mixtures containing the sample and 2 μCi of *S*-adenosyl[methyl-^{3}H]methionine (87 Ci/mmol) were pipetted into wells of a microtiter plate. They were incubated on ice and irradiated with 254-nm UV light in a Stratalinker 2400 cross-linking oven for 30 min. The distance of samples from the UV tubes was 4 cm. The samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

UV cross-linking of [α-^{32}P]GTP (20) was carried out in the same way. Twenty-five-microliter reaction mixtures contained 10 μCi of [α-^{32}P]GTP (400 Ci/mmol; final concentration, 1 μM). In competition experiments various amounts of unlabeled GTP or CTP were present. GTP at 1 mM inhibited the cross-linking by 80%, while 1 mM CTP had no effect. After irradiation SDS was added to a final concentration of 1%, and the samples were boiled, immunoprecipitated with anti-nsP1 antiserum, and analyzed by SDS-PAGE and autoradiography.

Other methods. Immunoprecipitation of SDS-denatured proteins and Western blotting (immunoblotting) by enhanced chemiluminescence (ECL; Amersham) were performed as described previously (13, 23). Western blots were quantitated with an Ultrascan XL enhanced laser densitometer (LKB); for ^{32}P-labeled samples a Phosphorimager (Molecular Dynamics) was employed.

Protein secondary structure predictions were generated by the neural network method of Rost and Sander (27, 28), which is more than 70% accurate, independently for three sets of proteins: (i) alphavirus nsP1 (8 different sequences available), (ii) N-terminal part of bromovirus 1a (8 sequences), and (iii) N-terminal part of potexvirus replicase (10 sequences).

Radioactive chemicals. 7-Methyl-GTP, which was ^{14}C-labeled at the 7-methyl group, [^{14}C]m^{7}GTP, was synthesized in the laboratory of Harri Lönnberg at the University of Turku (Turku, Finland) from GTP and [^{14}C]methyl iodide (Amersham) (7). Specific activity of the preparation was 50 mCi/mmol. Other radiolabeled compounds were from Amersham.

RESULTS

Construction of point mutations. Figure 1 shows a sequence comparison of the amino-terminal regions of SFV nsP1 (total of 537 amino acids [aa]) and two distantly related plant virus proteins, brome mosaic virus (BMV) protein 1a and potato virus X (PVX) replicase protein. Other parts of these proteins lack discernible similarities. According to the alignment, there are only four invariant amino acid residues (H38, D90, R93, and Y249 in SFV nsP1). SFV nsP1 and BMV 1a are more closely related to each other than to the PVX replicase, with several additional residues conserved between these two proteins, including D64, C135, and C142. As the sequence similarity is very low, there are likely to be regions where the alignment is incorrect. Also included in Fig. 1 are secondary

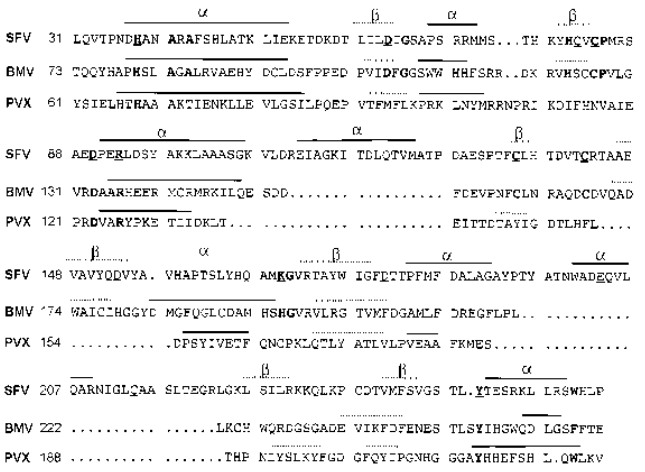

FIG. 1. Amino acid sequence comparison of SFV nsP1 (aa 31 to 262), BMV 1a (aa 73 to 262), and PVX replicase protein (aa 61 to 226) (combined and modified from references 1 and 29). The number of the first amino acid on each line is given on the left. Residues conserved between virus families are shown in boldface; note that all sequences from the alphavirus-like superfamily have been compared in making this alignment, and thus there are only few conserved residues. Amino acids mutated in the course of this study in SFV nsP1 are underlined. Secondary structure elements predicted by the method of Rost and Sander (27, 28) with a certainty of more than 50% are marked with lines (α helices) or dotted lines (β sheets) above each sequence.

structure predictions (27, 28), which show additional similarities between these proteins. They clearly belong to the α/β class of protein structures, which supports their relationship with cellular methyltransferases, as elaborated in the Discussion.

We selected the conserved residues for mutational analysis in order to discern their possible functional significance in SFV nsP1. Some additional point mutations were also generated for comparison. Thirteen of the mutations are located in the region shown in Fig. 1, where the respective residues are underlined. The properties of all 15 point mutants and three carboxy-terminally truncated forms of nsP1 are summarized in Table 1. The truncations were included in this analysis to define the region of nsP1 needed for enzymatic activities. Our recent work has shown that a deletion of 63 aa in the amino terminus of nsP1 produced enzymatically inactive protein (14). In most mutant proteins a single charged residue was changed into an alanine, as this change generally should not perturb the overall structure of the protein (4). In one mutant protein (L19E) a hydrophobic residue in the middle of a predicted alpha-helix was converted to glutamic acid. In this position a hydrophobic residue is found in all alphaviruses, and the mutation was therefore expected to be destructive for nsP1.

Expression and characterization of nsP1 derivatives. All nsP1 constructs were expressed in *E. coli*, as described previously for the wild-type protein (13). The recombinant proteins were easily detectable by Coomassie blue staining of the soluble fraction of bacterial extracts, as shown for three constructs in Fig. 2A. The relative amounts of nsP1 mutant proteins were estimated by Western blotting (Fig. 2B), followed by densitometric scanning. Three mutant proteins, C135A (Fig. 2B, lane 7), C142A, and D180A (not shown), were expressed repeatedly at somewhat lower levels. The lower band seen in Fig. 2B (lanes 1 to 8) represents either a premature termination or a carboxy-terminal degradation product of nsP1, since it is present in the construct 1-515 but absent from constructs containing larger C-terminal deletions (1-478; Fig. 2B, lane 9).

TABLE 1. Properties of nsP1 derivatives

Construct	Activity[a]		Cross-linking[b]	
	MT	GT	AdoMet	GTP
wt nsP1[c]	100	100	+	
No insert	<0.5	<0.5	−	−
L19E	<0.5	<0.5	−	(+)
H38A	160	<0.5	+	+
D64A	<0.5	<0.5	−	++
C81/83A	40	40	+	+
D90A	<0.5	<0.5	−	++
R93A	3	2	+	+
C135A	10	<0.5	(+)	+
C142A	<0.5	<0.5	−	+
D153A	100	100	+	+
K169A	50	100	+	+
D180A	100	ND	ND	ND
E203A	100	100	+	+
C214A	10	10	+	+
Y249A	3	<0.5	+	+
K317A	5	2	+	+
1-515	100	100	+	+
1-478	20	10	ND	+
1-429	<0.5	<0.5	ND	ND

[a] Activities are expressed as percentages of the wild-type level. MT assays were done in triplicate, and GT assays were done in duplicate. Assays with different batches of expression gave very similar results.

[b] +, wild-type level; −, negative; (+), detectable; ++, increased; ND, not determined.

[c] wt, wild type.

MT activity and covalent m^7GMP-nsP1 complex formation were assayed for all mutant proteins (Table 1 and Fig. 2C). The background, without nsP1 expression, in these assays was <0.5% of wild-type nsP1 activity ("No insert" in Table 1). Many of the mutants were either totally inactive in both reactions (L19E, D64A, D90A, and C142A) or had a very low level of activity (R93A, C135A, C214A, Y249A, and K317A). Most mutations, made in less conserved residues, showed wild-type (D153A, D180, and E203) or moderately reduced (C81/83A and K169A) activities. Interestingly, mutant H38A was totally deficient in the GT reaction (Fig. 2C, lane 3) but exhibited always a slightly increased MT activity (Table 1). Of the truncations, construct 1-515 was fully active, 1-478 had reduced activities, and 1-429 was inactive. Also, the truncation product

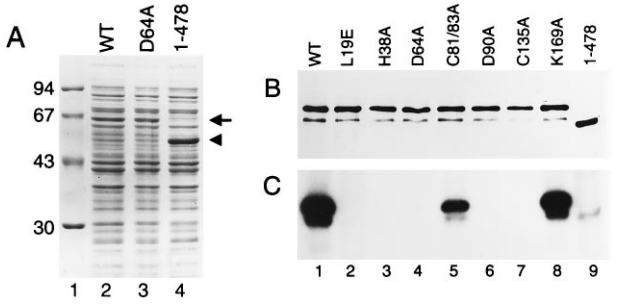

FIG. 2. Expression of nsP1 derivatives in *E. coli*. (A) Coomassie blue staining of the supernatant fractions of bacterial lysates. The arrow indicates the position of nsP1, and the arrowhead indicates the deletion 1-478. Molecular weight markers are shown on lane 1 with their sizes (in kilodaltons) on the left. (B) Western blot of the supernatant fractions of bacterial extracts, probed with anti-nsP1 antiserum. One-tenth the amount of samples shown in panel A was loaded. (C) The same extracts as in panel B were incubated with [α-^{32}P]GTP in the presence of 100 μM AdoMet and analyzed by SDS-PAGE and autoradiography to reveal the covalent guanylate complexes formed.

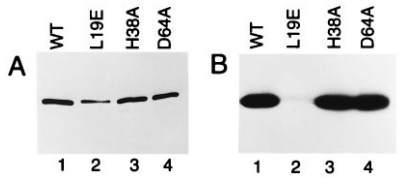

FIG. 3. Expression and palmitoylation of nsP1 derivatives in HeLa cells. (A) Western blot of HeLa cell extracts infected with vaccinia virus vTF7-3, transfected with nsP1 constructs, and labeled with ^{3}H-palmitate. The blot was probed with anti-nsP1 antiserum. (B) Equal portions of the same extracts immunoprecipitated with anti-nsP1, run in SDS-PAGE, and fluorographed to show the ^{3}H-palmitate labeling of nsP1 derivatives.

occurring in *E. coli* (Fig. 2B, lower band in lanes 1 to 8) was fully active in the GT reaction (Fig. 2C, lower band in lanes 1, 5, and 8). In length, it is between constructs 1-515 and 1-478. Thus, it seems that about 500 amino acids are needed for full enzymatic activities of nsP1.

The mutated forms of nsP1 were also synthesized in HeLa cells by transient transfection using a recombinant vaccinia virus vTF7-3 expression system (5). Palmitoylation of nsP1 variants was studied by labeling the cells with ^{3}H-palmitate and following labeling by immunoprecipitation with nsP1 antiserum. It was found that all mutated forms of nsP1 were labeled with palmitate (the truncations were not examined in this system). Some constructs, including C135A and C142A, were poorly expressed, but their palmitoylation was still readily detectable (not shown). However, most of the enzymatically inactive mutants were expressed and palmitoylated at levels comparable to wild-type nsP1 (Fig. 3). When the amount of palmitoylation was normalized by the level of expression, as estimated from Western blots, it was always close to the wild-type level (70 to 100%), with the exception of mutant L19E, which was palmitoylated very weakly (always less than 10% of the wild-type level; Fig. 3B, lane 2). It can be concluded that palmitoylation of nsP1 is not correlated with its enzymatic activity.

7-Methyl-GTP as a substrate for nsP1. As all MT negative mutants were also GT negative (Table 1), we wanted to measure the GT activity independently of methylation. For this purpose chemically synthesized [^{14}C]m^7GTP was used. In the absence of nsP1 expression, no reaction in the bacterial extract was detected (not shown). Unexpectedly, [^{14}C]m^7GTP did not react with nsP1 to form the covalent complex (Fig. 4, lane 1). Addition of AdoMet had no effect (lane 7), and the mutated forms of nsP1 also gave negative results. However, when [^{14}C]m^7GTP was incubated with nsP1 in the presence of AdoHcy, ^{14}C-labeled nsP1 was synthesized (lane 2). The same result was obtained with all nsP1 mutants, which were active in

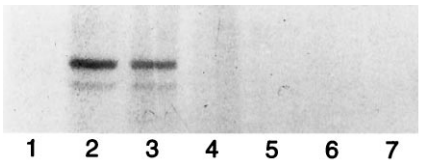

FIG. 4. Covalent guanylate complex formation of nsP1 derivatives with [^{14}C]m^7GTP. Extracts from *E. coli* expressing nsP1 or its mutated forms were incubated with 250 μM [^{14}C]m^7GTP in GT assay buffer (see Materials and Methods), either in the absence of additives or in the presence of 100 μM AdoHcy or AdoMet, as indicated below. Reactions were stopped by boiling in 2% SDS, and equal amounts were analyzed by SDS-PAGE and fluorography. Lanes: 1, wild-type nsP1; 2, wild-type nsP1 plus AdoHcy; 3, C81/83A plus AdoHcy; 4, D64A plus AdoHcy; 5, R93A plus AdoHcy; 6, H38A plus AdoHcy; 7, wild-type nsP1 plus AdoMet.

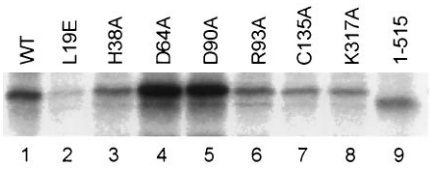

FIG. 5. UV light-induced cross-linking of nsP1 derivatives with [α-^{32}P]GTP. Extracts from bacteria expressing the nsP1 derivative indicated above each lane were incubated on ice in a UV cross-linking oven for 30 min in the presence of EDTA and 1 μM [α-^{32}P]GTP (see Materials and Methods). The reaction mixtures were subjected to immunoprecipitation with anti-nsP1 antibodies after denaturing of proteins with SDS. Equal portions of these samples were analyzed by SDS-PAGE and autoradiography.

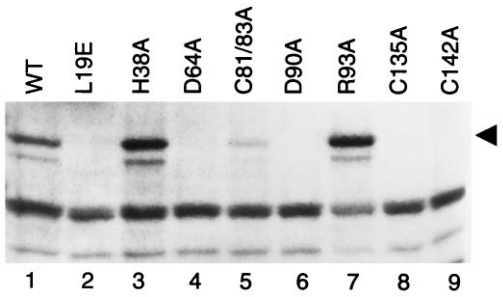

FIG. 6. UV light-induced cross-linking of nsP1 derivatives with *S*-adenosyl-[methyl-^{3}H]methionine. Arrowhead indicates the position of nsP1. Extracts from bacteria expressing the nsP1 derivative indicated above each lane were incubated on ice in a UV cross-linking oven for 30 min in the presence of EDTA and tritiated AdoMet (see Materials and Methods). Reactions were stopped by boiling in 2% SDS, and equal amounts were analyzed by SDS-PAGE and fluorography.

both MT and GT reactions (as demonstrated for mutant C81/83A in Fig. 4, lane 3), but not with mutants inactive in either MT (D64A and R93A; lanes 4 and 5, others not shown) or GT (H38A; lane 6) reactions.

Thus, MT activity seemed to be required for the GT reaction in the presence of [^{14}C]m^{7}GTP and AdoHcy. We assume that nsP1 catalyzes first the demethylation of [^{14}C]m^{7}GTP, giving rise to GTP and *S*-adenosyl[methyl-^{14}C]methionine. Then in a forward reaction GTP is again methylated and forms the labeled covalent complex m^{7}GMP-nsP1. The alternative explanation that AdoHcy allosterically facilitates complex formation between nsP1 and m^{7}GTP is less likely, since all MT-negative mutants failed to carry out this reaction. In any case, the implication for the reaction mechanism of nsP1 is that methylation and complex formation are tightly coupled within the protein.

Cross-linking of nsP1 with GTP and AdoMet. Next, we wanted to know whether substrate binding was specifically defective in the numerous MT-negative mutants. To this end, we used UV irradiation to cross-link radioactively labeled GTP and AdoMet separately with nsP1 and its derivatives. These reactions were carried out in the presence of EDTA to chelate divalent cations. Under these conditions nsP1 is active as an MT (13), but the GT reaction is totally inhibited (2).

UV cross-linking of [α-^{32}P]GTP to nsP1 was relatively weak, necessitating immunoprecipitation of nsP1 from the reaction mixture. It was specific, since it could be inhibited by 80% with unlabeled 1 mM GTP but not with CTP. GTP could be cross-linked to all point-mutated forms of nsP1, as well as to deletions 1-515 and 1-478 (Table 1; examples are shown in Fig. 5), but with variable efficiency. The weakest cross-linking was to the mutant protein L19E (Fig. 5, lane 2). Strongest cross-linking observed was to D64A and D90A (lanes 4 and 5). Other mutant proteins gave results similar to those of the wild-type protein (lanes 1, 3, and 6 to 9).

Most nsP1 derivatives were cross-linked also to AdoMet, as shown in Fig. 6 and summarized in Table 1, including the weakly active mutants R93A, C214, Y249A, and K317A. nsP1 was the major protein cross-linking with AdoMet in bacterial extracts. In the absence of nsP1 expression, no labeled protein this size was observed, and the cross-linked complex could be precipitated by anti-nsP1 antibodies (not shown). The smaller labeled proteins represent AdoMet-binding proteins of *E. coli*, serving as internal controls (positive in all lanes). Mutant protein C135A showed very weak cross-linking (lane 8; can be seen only in the original autoradiograph), whereas cross-linking to mutants L19E (lane 2), D64A (lane 4), D90A (lane 6), and C142A (lane 9) was undetectable. These four mutant proteins also lacked MT activity (Table 1). Cross-linking to protein C81/83A was relatively weak (lane 5), whereas other point-mutated proteins showed wild-type efficiency.

Mutation L19E may create a serious conformational disturbance in nsP1 because of the change of a conserved hydrophobic residue to a charged one. The defect was reflected also in the low efficiency of cross-linking with GTP as well as very low level of palmitoylation (Fig. 3). Derivatives C142A and C135A were expressed at a lower level, and thus these cross-linking results may not be entirely comparable with the others. Taken together, our results suggest that the first 150 amino acids of nsP1 are important for AdoMet binding and residues D64 and D90 (possibly also C142 and C135) have critical roles in this interaction. It seems that GTP-binding residues are less conserved in this family of proteins than those involved in nsP1-AdoMet interaction, since all mutant proteins retained the GTP cross-linking capacity (Fig. 5).

DISCUSSION

We have shown that SFV nsP1 has two enzymatic activities: MT and an ability to form a covalent complex with m^{7}GMP, which corresponds to the first reaction catalyzed by GTs (2, 13). Here we attempted to correlate these reactions with amino acid residues, which are conserved in the alphavirus-like superfamily, by using as tools site-directed mutagenesis of SFV nsP1 and secondary structure predictions (Fig. 1). When the enzymatic activities of the mutant proteins were assayed (Table 1), it was observed that the GT reaction was strictly dependent on MT activity. When MT activity was absent or severely reduced, the GT reaction also was absent or reduced. These results support our previous scheme of alphavirus mRNA capping, in which methylation of GTP is required for covalent complex formation (2). This mode of capping is strictly virus specific and should offer a possibility for designing specific inhibitors of alphavirus replication. Our present results indicate also that in the reaction mechanism of nsP1, the MT and GT reactions are tightly coupled, since exogenously added m^{7}GTP could not react directly to form the covalent complex (Fig. 4).

One mutation (H38A) selectively destroyed the GT reaction and slightly increased the MT activity, indicating that this absolutely conserved histidine residue was required only for GT activity. It is tempting to speculate that H38 would be the covalent binding site of m^{7}GMP, since specific hydrolysis conditions indicated that the nucleotide is bound to nsP1 via a phosphoamido linkage (2). Interestingly, mutation of the corresponding histidine (H39) to glutamine in Sindbis virus nsP1 resulted in noninfectious virus RNA (19). As residues critical for both enzymatic activities are conserved in the large alpha-

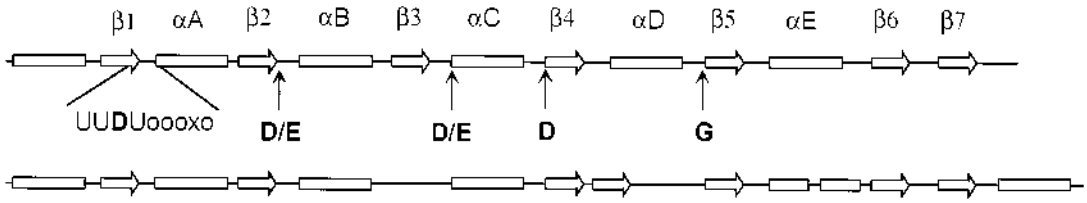

FIG. 7. Comparison of secondary structures. Arrows are β sheets, and boxes represent alpha-helices. The top portion shows the general structure of AdoMet-dependent MTs, where elements are numbered according to Schluckebier et al. (32). Conserved amino acid residues or sequences are marked in appropriate locations. Standard one-letter abbreviations for amino acids are used. U, large hydrophobic residue; o, small residue (G, A, or S); x, any residue. The bottom portion shows the predicted secondary structure of SFV nsP1 (for the conserved region only; see Fig. 1).

virus-like superfamily, which includes both animal and plant pathogens, it seems probable that they all perform unique capping reactions like that performed by the alphaviruses. This should increase the motivation to search for specific capping inhibitors.

Recently, Wang et al. (36) described six point mutations of Sindbis virus nsP1 and their effect on MT activity in *E. coli*. Four of their mutants were targeted to the absolutely conserved residues (Fig. 1) and caused loss of MT activity. However, we could detect a small residual activity with R93A and Y249A mutants. Still, there remains a serious discrepancy with mutant H38A, which in our experiments destroyed only the GT reaction. This difference is not due to the different methyl acceptor used in our assays, since we obtained essentially the same results with the guanylylimidodiphosphate used by Wang et al. (data not shown). The discrepancy is difficult to explain, but it should be noted that all constructs examined by Wang et al. (36) contained, in addition to the new mutations, the double mutation R87L-S88C (17). The authors showed also that mutations in the four conserved residues abolished infectivity when transferred to the virus genome, implying that capping of viral mRNAs is necessary for successful virus replication.

The recent elucidation of three-dimensional structures of five AdoMet-dependent MTs has revealed remarkable similarity in AdoMet-binding regions, which contain also the catalytic residues. This region constitutes a domain of ca. 200 aa (not necessarily continuous in amino acid sequence [16]) with a central antiparallel β sheet flanked by alpha-helices (32). The amino acid sequences of different methyltransferases are widely variable, and even for the most conserved sequence element, only a very loose consensus sequence G-loop can be defined (Fig. 7) (10, 12). Structure-guided analyses have revealed additional single amino acid residues often conserved in MTs (16, 21). The G-loop (after β-strand 1) and the loops after β-strands 2 and 3 interact with AdoMet. Mutational analysis of a DNA methyltransferase (*Eco*KI) has demonstrated the importance of the G-loop in AdoMet-binding (38). The loop following β-strand 4 participates in this interaction. It also contains the most important catalytic residues (3, 9, 15, 32).

Our secondary structure predictions of nsP1 and related plant virus proteins (Fig. 1) suggested that these viral MTs might share a similar structure. A linear diagram comparing the secondary structure elements of known MT structures (32) with those predicted for nsP1 is shown in Fig. 7. The predictions used for the three very distantly related groups of viral proteins (alphavirus nsP1s and related domains from bromoviruses and potexviruses) included quite similar results. There were also dissimilarities, like loops or additional structure elements (Fig. 1). However, the beginning of the conserved domain does not contain insertions and can be interpreted to represent a structural unit, α-β-α-β-α. nsP1 contains a conserved sequence element, ILDIGSAPS (aa 62 to 70), which fits to the G-loop consensus. Sequences fitting the consensus are also present in more closely related virus proteins (BMV 1a, Fig. 1) but replaced by a conserved hydrophobic-positive motif in more distantly related ones (PVX replicase in Fig. 1). According to the secondary structure predictions, these sequence motifs can be placed into a position corresponding to the G-loop (Fig. 7).

One motivation for the site-directed mutational analysis was to find support for our hypothesis that nsP1 is structurally related to other MTs. Several of the nsP1 mutant proteins had lowered MT activity (Table 1), while four were completely negative. Proteins D64A and D90A were unable to bind AdoMet, as assayed by UV cross-linking. Remarkably, D64A has a mutation in the conserved acidic residue of the proposed G-loop of SFV nsP1. D90 could well correspond to the acidic residue, commonly found after β-strand 2, which is involved in binding of the ribose hydroxyls of AdoMet (32). The conserved R93 is crucial for MT activity but not for AdoMet binding (Table 1). The mutations of other proteins defective in AdoMet binding (C81/83A, C135A, and C142A, with the exception of L19E) mapped also to the region corresponding to the AdoMet-binding domain of cellular MTs. The conclusion that this part of the protein binds AdoMet is further supported by a finding on Sindbis virus nsP1. A double mutant R87L-S88C (corresponding to R86 and S87 in SFV nsP1), selected in cell culture under low-methionine conditions, had increased MT activity (17, 31).

Thus, experimental evidence and secondary structure predictions support the idea that at least the amino-terminal part of the conserved region in nsP1-related proteins corresponds to the known MTs. The putative relationship of these proteins with cellular MTs is a novel observation, interesting especially from an evolutionary point of view. These RNA viruses seem to have retained the basic structure of a cellular MT, in spite of a high mutation rate during their genome replication.

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