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Role of myristoylation of poliovirus capsid protein VP4 as determined by site-directed mutagenesis of its N-terminal sequence

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Mutations were introduced by oligonucleotide-directed mutagenesis into the cDNA of poliovirus type 1 (Mahoney) in the region coding for the first five amino acids (myristoylation signal) of the viral capsid protein precursor P1. The cDNAs were then transcribed *in vitro* and the properties of the transcripts carrying the mutations studied *in vitro* by translation in a reticulocyte lysate or *in vivo* upon transfection of primate cells. Mutation of amino acid residue number 5 (Ser5 → Thr) did not affect the viral phenotype, whereas mutations of residues number 1 (Gly1 → Arg), 2 (Ala2 → Pro) or 5 (Ser5 → Pro) prevented myristoylation of P1 and were lethal. However, delayed production of virus was occasionally observed as the result of reverse mutations, which were found to restore a functional myristoylation signal as well as a wild-type virus phenotype. Thus, the myristoylation signal of the poliovirus polyprotein can accommodate Ala, Ser, Thr or Leu residues at position 2 and Ser, Thr or Ala residues at position 5. Mutations that altered myristoylation of P1 and affected virus viability did not prevent replication of the viral RNA but severely impeded *in vitro* processing of P1. This suggests that myristoylation plays a role in poliovirus capsid protein assembly.

Key words: capsid/mutation/myristoylation/poliovirus/processing

Introduction

Poliovirus, a member of the Picornaviridae family, is a non-enveloped icosahedral virus consisting of 60 copies each of four capsid proteins (VP1, VP2, VP3 and VP4) and a single-stranded RNA genome of positive polarity (for review see Rueckert, 1985). Translation of the genomic RNA generates a 247 000 dalton polyprotein (NCVP00) which is processed into three precursors, P1, P2 and P3, given in the N- to C-terminal order (Kitamura *et al.*, 1981). Precusor P1 is cleaved into three structural proteins, VP0, VP3 and VP1, which form the 5S protomer (Bruneau *et al.*, 1983). During viral assembly, five protomers give rise to a 14S pentamer. Twelve of these pentamers assemble to form a provirion while incorporating the genomic RNA (Putnak and Phillips, 1981; Rueckert, 1985). During maturation of the virion, which is the latest step of virus assembly, VP0 is processed to VP4 and VP2, probably by an autocatalytic process (Putnak and Phillips, 1981; Arnold *et al.*, 1987). VP4 as well as its precursors VP0 and P1 are myristoylated (Chow *et al.*, 1987; Paul *et al.*, 1987) at their N-terminal glycine by an amide linkage. This covalent modification is a co-translational event (Wilcox *et al.*, 1987) that occurs after removal of the methionine residue initiating the viral polyprotein.

The three-dimensional structure of the poliovirion was determined at a resolution of 2.9 Å (Hogle *et al.*, 1985).

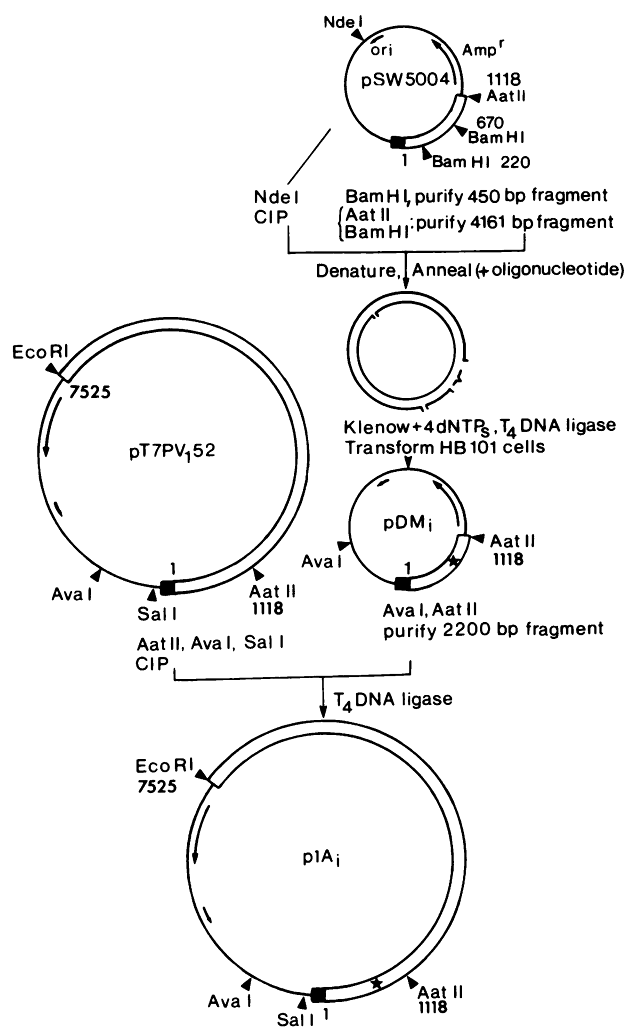

Fig. 1. Oligonucleotide site-directed mutagenesis. Mutagenesis was carried out on plasmid pSW5004 as described in Materials and methods. To reintroduce the mutation into full-length poliovirus cDNA, the 2.2 kb *AvaI–AatII* fragments of plasmids pDMi, harboring the mutations (*), were purified and individually recombined with the 9.3 kb *AvaI–AatII* fragment of pT7 PV1-52, yielding plasmids p1Ai (i referring to the number of the synthetic oligonucleotide used for mutagenesis). (■) ϕ10 T7 promoter. (—) pBR322. (□) PV1 cDNA. Numbering refers to nucleotide positions in the PV1 sequence.

VP1 is located closed to the 5-fold axis, whereas VP2 and VP3 alternate around the 3-fold axis of the icosahedron. Capsid protein VP4 represents an amino-terminal extension of VP2, located at the inner face of the virus shell. The myristate moiety of VP4 is located close to the 5-fold axis of the virus particle, and there takes part, together with the 5-fold-related N termini of VP4 and VP3, in a unique structure known as a β-tube (Chow *et al.*, 1987).

An increasing number of eukaryotic proteins of either viral (Henderson *et al.*, 1983; Persing *et al.*, 1987; Streuli and Griffin, 1987) or cellular (Aitken *et al.*, 1982; Carr *et al.*, 1982) origin have been shown to be myristoylated. Analysis of their N-terminal sequence as well as studies making use of a series of synthetic oligopeptides as substrates for N-myristoyl glycine transferase (NMT) purified from yeast (Towler *et al.*, 1987b) or rat liver (Towler *et al.*, 1988a) have demonstrated that the presence of an N-terminal Gly residue to which the fatty acid moiety is attached is essential at position 1, while Ser, Thr or Ala residues are usually found at position 5. In addition, some restrictions seem to exist for residues at positions 2 and 6 (for a review, see Towler *et al.*, 1988b).

We have investigated the possible function of the myristoyl group linked to the N terminus of poliovirus capsid protein VP4 and of its precursor VP0 and P1. Point mutations were generated in the nucleotide sequence coding for VP4 in order to modify the N terminus of the polyprotein (Met0 Gly1 Ala2 Gln3 Val4 Ser5 Ser6) so as to affect its myristoylation. Mutations were introduced by oligonucleotide site-directed mutagenesis into a cDNA clone (pT7 PV1-51) of poliovirus type 1 (PV1) which can be transcribed *in vitro* into an infectious genomic RNA (van der Werf *et al.*, 1986). The effect of the mutations was studied both *in vitro* in a cell-free translation system and *in vivo* after transfection of cells permissive for poliovirus replication.

We demonstrate here that myristoylation of VP4 and/or of its precursors is an absolute requirement for multiplication of the virus. We further show that mutations that prevent myristoylation seem to alter proper processing of the precursor P1 protein *in vitro* and thus might hinder virus assembly.

Results

Site-directed mutagenesis of the myristoylation sequence of VP4

In an attempt to determine the function of the myristic acid moiety linked at the N terminus of poliovirus capsid polypeptide VP4, point mutations were introduced in the region of the viral cDNA coding for the putative myristoylation signal of VP4 (Met0 Gly1 Ala2 Gln3 Val4 Ser5 Ser6). Thus, mutation Ser5 → Thr, which should not affect myristoylation of VP4, and mutations Gly1 → Arg, Ala2 → Pro and Ser5 → Pro, which should prevent it (Towler *et al.*, 1988b), were introduced by oligonucleotide site-directed mutagenesis according to Morinaga *et al.* (1984) (Figure 1). Mutagenesis was performed on plasmid pSW 5004 which contains only the first 1118 nt of the poliovirus cDNA (see Materials and methods). The mutations were next reintroduced into a genomic cDNA, generating plasmid pG1-1A1 to pG1-1A4 (abbreviated p1A1 to p1A4 throughout the text), named according to the nomenclature proposed by Bernstein *et al.* (1986) where 1A designates VP4 (Rueckert and Wimmer, 1984), and the following number the synthetic oligonucleotide used in the mutagenesis. As shown in Table I, the oligonucleotides were chosen such as to modify one or two codons and create simultaneously a new restriction site in the cDNA.

Table I. Sequences of the oligonucleotides used for mutagenesis

		0	1	2	3	4	5	6	7	8	9
		Met	Gly	Ala	Gln	Val	Ser	Ser	Gln	Lys	Val
PV1	GT ATC ATA	ATG	GGT	GCT	CAG	GTT	TCA	TCA	CAG	AAA	GTG
		Met	**Arg**	Ala	Gln	Val	Ser	Ser	Gln	Lys	Val
#1	GT ATC ATA	ATG	**C**G**C**	GCT	CAG	GTT	TCA				
		BssH II (ATG CGC GC)									
		Met	Gly	Ala	Gln	Val	**Thr**	Ser	Gln	Lys	Val
#2				GCT	CAG	GTT	**ACC**	TCA	CAG	AAA	GTG
					BstE II (G GTT ACC)						
		Met	Gly	Ala	Gln	Val	**Pro**	Ser	Gln	Lys	Val
#3		G	GGT	GCT	CAG	GT**A**	**C**CA	TCA	CAG	AAA	G
					Kpn I (G GTA CC)						
		Met	Gly	**Pro**	Gln	Val	Ser	Ser	Gln	Lys	Val
#4	C ATA	ATG	GGT	**C**CT	CAG	GTT	TC				
				Mst II/Sau I (CCT CAG G)							

The nucleotide sequence of PV1 cDNA and the N-terminal amino acid sequence of the polyprotein are shown on top. For each of the four oligonucleotides, nucleotides and amino acids that were modified by mutagenesis are indicated in bold characters and the resulting new restriction sites are underlined.

Lack of myristoylation affects virus viability

To study the effects of the mutations on virus viability, plasmids pT7 PV1-51 and p1A1 to p1A4 were linearized by *Eco*RI and transcribed *in vitro* using T7 RNA polymerase. The specific infectivity of the genomic transcripts was determined upon transfection of HeLa or VERO cells (see Materials and methods). Whereas transcripts from pT7 PV1-51 and p1A2 were infectious (specific infectivity = 10^5 p.f.u./μg), transcripts from p1A1, p1A3 and p1A4 were not (specific infectivity <10 p.f.u./μg). This was observed in HeLa and VERO cells, and was independent of the temperature (33,37 or 39°C) (data not shown). Thus, mutation 1A2 (Ser5 → Thr) did not alter virus viability, whereas the three other mutations were lethal.

To determine whether mutations 1A1 to 1A4 affect myristoylation of VP4 and of its precursor VP0 and P1 *in vivo*, HeLa cells were transfected with each of the mutated full-length transcripts and labeled from 3 to 7.5 h post-transfection either with [^{35}S]methionine or with [^{3}H]myristate. The ^{35}S- and ^{3}H-labeled proteins were then extracted, immunoprecipitated with an αC (directed against heat-treated poliovirus particles) or an αVP2 rabbit serum respectively, and analyzed by SDS−PAGE. As shown in Figure 2A, ^{35}S-labeled VP0 was present in all extracts from cells transfected with either infectious or non-infectious transcripts. In contrast, as shown in Figure 2B, ^{3}H-labeled VP0 was readily detected only in extracts of cells transfected with viral RNA, wild-type pT7 PV1-51 transcripts or p1A2 transcripts. A faint band of ^{3}H-labeled VP0 could also be seen after transfection with p1A3 or p1A4 transcripts. However, no ^{3}H-myristate labeling could be detected after transfection with p1A1 transcripts. Therefore, while mutation 1A2 did not affect the myristoylation of VP0, mutations 1A3 and 1A4 severely impaired it and mutation 1A1 totally prevented it. These results strongly suggest that myristoylation of VP4 and/or of its precursors is an absolute

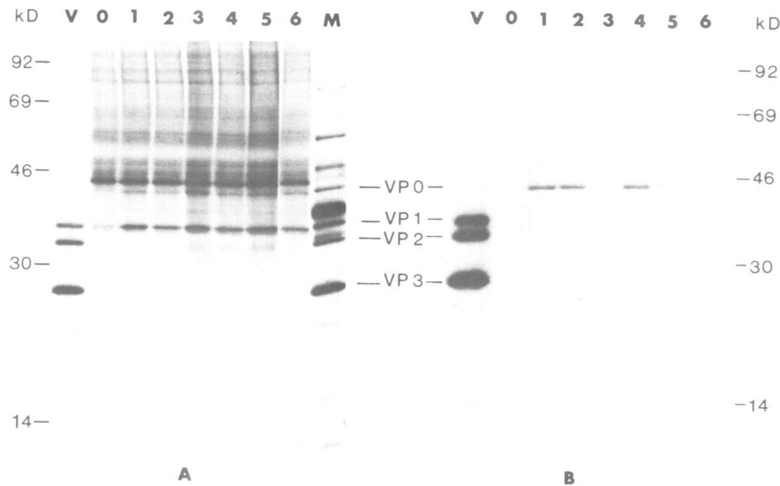

Fig. 2. Myristoylation of mutated VP0 *in vivo*. HeLa cells (10^6) were mock transfected (**lanes 0**) or transfected with 0.05 μg of purified virion RNA (**lanes 1**), or with ~1 μg of full-length transcripts from pT7 PV1-51 (**lanes 2**) or from p1A1−p1A4 (**lanes 3−6**). They were labeled with [^{35}S]methionine (**panel A**) or [^{3}H]myristic acid (**panel B**) from 3 to 7.5 h post-transfection and cytoplasmic extracts were prepared (see Materials and methods). One-half of each extract was immunoprecipitated either with an αC serum (**panel A**) or with an αVP2 serum (**panel B**). The immune precipitates were analyzed by SDS−PAGE. The radioactive protein bands were visualized on Fuji X-ray film after 7 days of exposure (**panel A**) or on Hyperfilm MP (Amersham) after 50 days of exposure (**panel B**). V, [^{35}S]methionine-labeled PV1; M, [^{35}S]methionine-labeled cytoplasmic extract from PV1-infected HeLa cells. Viral capsid polypeptides are indicated in the middle.

requirement for multiplication of the virus. This conclusion was further supported by the analysis of reversions of mutations 1A1, 1A3 and 1A4 (see below).

Analysis of viruses resulting from transfection with mutated transcripts

HeLa cells were transfected with each of the infectious or non-infectious transcripts and the appearance of a cytopathic effect (CPE) was monitored at 37°C (see Materials and methods). Transfection with purified viral RNA, with transcripts from pT7 PV1-51 or with p1A2 transcripts, was followed 1 day later by the appearance of CPE. In the case of p1A1, p1A3 and p1A4 transcripts, which were not infectious in titration experiments, transfection into HeLa cells occasionally led to viral production and CPE. This was a late (3−5 days) and sporadic event, occurring in 3 out of 10 plates transfected with p1A1 transcripts, in 9 out of 10 plates transfected with p1A3 transcripts and in all the plates transfected with p1A4 transcripts.

Individual viral ministocks were prepared from each plate where CPE was evident, and the sequence of the viral RNA in the region coding for the amino terminus of VP4 was determined. Table II shows that the genome of the virus resulting from transfection with p1A2 transcripts had retained the original codon from p1A2, thus confirming the observation that mutation Ser5 → Thr is viable. However, in the case of non-infectious p1A1, p1A3 and p1A4 transcripts, sequencing of the viral RNA revealed that viral production was the result of point mutations specifically involving the codon that had originally been modified by site-directed mutagenesis.

Thus, the three viruses born from transfection with p1A1 transcripts had all reverted to a GGC (Gly1) codon from the CGC (Arg1) codon present on plasmid p1A1. This CGC codon could have been equally modified by point mutations to AGC (Ser), UGC (Cys), CAC (His), CCC (pro) or CUC (Leu) but none of these reversions were observed. This indicates that none of these amino acids are permitted at position 1 of the VP4 sequence, suggesting that a Gly residue is required at that position for the protein to be myristoylated and for the virus to be viable.

After transfection with p1A3 transcripts three groups of

Table II. Sequences of the myristoylation revertants

cDNA/RNA	Myristoylation signal							frequency (1)
	0	1	2	3	4	5	6	
PV1 RNA								
pT7 PV1-51	Met	Gly	Ala	Gln	Val	Ser	Ser	
vT7 PV1-51	ATG	GGT	GCT	CAG	GTT	TCA	TCA	
p1A1	Met	**Arg**	Ala	Gln	Val	Ser	Ser	
	ATG	**CGC**	GCT	CAG	GTT	TCA	TCA	
v1A100	Met	Gly	Ala	Gln	Val	Ser	Ser	3/3
	ATG	G**GC**	GCT	CAG	GTT	TCA	TCA	
p1A2	Met	Gly	Ala	Gln	Val	**Thr**	Ser	
	ATG	GGT	GCT	CAG	GTT	**ACC**	TCA	
v1A200	Met	Gly	Ala	Gln	Val	**Thr**	Ser	
	ATG	GGT	GCT	CAG	GTT	**ACC**	TCA	
p1A3	Met	Gly	Ala	Gln	Val	**Pro**	Ser	
	ATG	GGT	GCT	CAG	GT**A**	**C**CA	TCA	
v1A301	Met	Gly	Ala	Gln	Val	**Ala**	Ser	1/9
	ATG	GGT	GCT	CAG	GT**A**	**G**CA	TCA	
v1A302	Met	Gly	Ala	Gln	Val	**Thr**	Ser	6/9
	ATG	GGT	GCT	CAG	GT**A**	**A**CA	TCA	
v1A305	Met	Gly	Ala	Gln	Val	Ser	Ser	2/9
	ATG	GGT	GCT	CAG	GT**A**	TCA	TCA	
p1A4	Met	Gly	**Pro**	Gln	Val	Ser	Ser	
	ATG	GGT	**C**CT	CAG	GTT	TCA	TCA	
v1A401	Met	Gly	**Thr**	Gln	Val	Ser	Ser	4/12
	ATG	GGT	**A**CT	CAG	GTT	TCA	TCA	
v1A403	Met	Gly	Ala	Gln	Val	Ser	Ser	3/12
	ATG	GGT	GCT	CAG	GTT	TCA	TCA	
v1A405	Met	Gly	**Leu**	Gln	Val	Ser	Ser	4/12
	ATG	GGT	**CT**T	CAG	GTT	TCA	TCA	
v1A406	Met	Gly	**Ser**	Gln	Val	Ser	Ser	1/12
	ATG	GGT	**T**CT	CAG	GTT	TCA	TCA	

The nucleotide and deduced amino acid sequences of the viral polyprotein are shown for the indicated plasmids (p) or for the corresponding viruses (v) recovered upon transfection of HeLa cells with the genomic transcripts derived from these plasmids. Mutated nucleotides and amino acid residues are indicated in bold characters. (1) Frequency of occurrence of each of the revertant viruses.

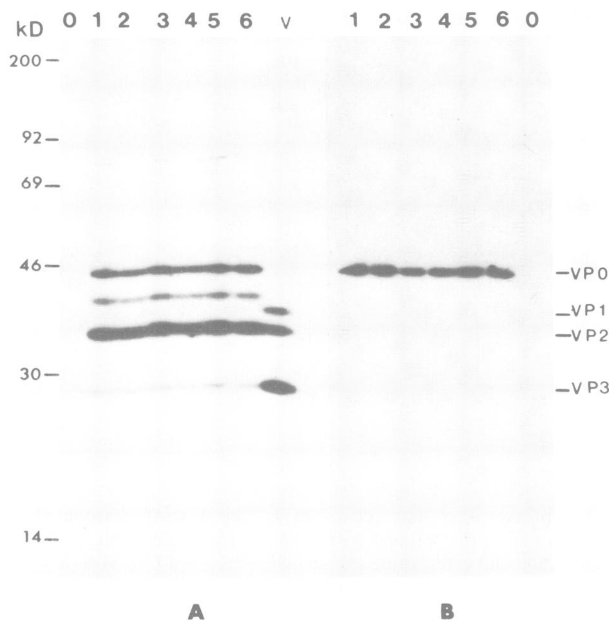

Fig. 3. Myristoylation of VP0 for the revertant viruses. Hela cells (10^6) were mock infected (**lane 0**) or infected with vT7 PV1-51, v1A200, v1A301, v1A401, v1A405 or v1A406 (**lanes 1–6** respectively) and labeled with [^{35}S]methionine (**panel A**) or [^{3}H]myristate (**panel B**) as described in Materials and methods. The labeled proteins were immunoprecipitated with an αVP2 serum and analyzed by SDS–PAGE. The radioactive protein bands were visualized on Fuji X-ray film after overnight (**panel A**) or 3 days (**panel B**) of exposure. V, [^{35}S]methionine-labeled PV1. Viral capsid polypeptides are indicated on the right.

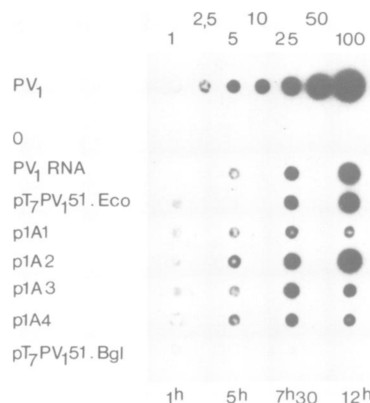

Fig. 4. Replication of mutated transcripts. HeLa cells (10^6) were mock transfected or transfected with 0.05 μg of PV1 RNA, or with ~1 μg of genomic transcripts from the indicated plasmids linearized with *Eco*RI (pT7 PV1-51.Eco, p1A1–p1A4), or with ~ 1 μg of a subgenomic transcript from pT7 PV1-51 linearized with *Bgl*II (pT7 PV1-51.Bgl). At the indicated times after transfection, cytoplasmic RNAs were extracted, bound to a nylon membrane and then hybridized to a ^{32}P-labeled riboprobe complementary to nt 3417–4830 of the viral RNA (see Materials and methods). PV1, increasing amounts of purified viral RNA (1–100 ng) were hybridized in parallel.

viruses were recovered. They all resulted from a mutation in the CCA (Pro5) codon, which was changed to **G**CA (Ala5), **U**CA (Ser5) or **A**CA (Thr5). Mutation CCA → **A**CA was the most frequently observed, whereas reversion CCA → **U**CA restored the wild-type genotype. Among the three other possible reversions C**U**A (Leu), C**A**A (Gln) and C**G**A (Arg), none was observed, suggesting that they did not restore virus viability.

After transfection with p1A4 transcripts, four groups of viruses were recovered. They resulted from a modification of the CCU (Pro2) codon to **A**CU (Thr2), **U**CU (Ser2), **G**CU (Ala2) or C**U**U (Leu2). Reversions to C**A**U (His) and C**G**U (Arg) were not observed, suggesting that these two amino acids at position 2 would affect virus viability.

The result of the reversions was to restore myristoylation of VP4 and its precursors. This was shown by infecting HeLa cells with each of the revertant viruses and labeling them with either [^{35}S]methionine or [^{3}H]myristate. Labeled proteins were immunoprecipitated with an αVP2 serum prior to analysis by SDS–PAGE (Materials and methods). As can be seen in Figure 3A, in cells infected with the revertant viruses, the amount of VP0 and VP2 was equivalent to that observed in cells infected with wild-type virus. As shown in Figure 3B, myristoylation of VP0 occurred with equal efficiency in all cases. Therefore, whereas mutations Gly1 → Arg, Ala2 → Pro and Ser5 → Pro were shown to affect myristoylation of VP0 and to be lethal, the spontaneous reversion of these mutations restored both myristoylation of VP0 and viability of the virus. The revertant viruses were titrated on HeLa and VERO cells at 33, 37 and 39°C and they exhibited neither temperature-sensitive nor host-range phenotype (data not shown).

Effect of the mutations on the replication of viral RNA

To investigate at which step virus multiplication was blocked by the lack of myristoylation of VP4 and of its precursors, we first analyzed the ability of the mutated transcripts to replicate in permissive cells. To that end, HeLa cells were transfected with the wild-type and mutated transcripts, as well as with an equivalent infectious dose of purified viral RNA. At various times post-transfection, cytoplasmic RNAs were extracted, immobilized on a nylon membrane and hybridized with a ^{32}P-labeled riboprobe complementary to nt 3417–4830 of the viral RNA. As shown in Figure 4, the hybridization signal increased from 1 to 7.5 h after transfection with purified viral RNA or with transcripts of genomic length, irrespective of the mutations in the VP4 sequence, while no hybridization could be detected with cytoplasmic RNA from mock-transfected cells (lane 0) or from cells transfected with a subgenomic transcript lacking the nucleotide sequences 3′ of nt 5601 of the viral RNA (bottom lane, pT7 PV1-51 Bgl). These results unambiguously demonstrate that all the genomic transcripts, whether infectious or not, were able to replicate in the transfected cells. However, beyond 7.5 h post-transfection, the hybridization signal significantly increased only in the case of transfection with infectious RNAs, while it tended to decrease in the case of transfection with non-infectious p1A1, p1A3 or p1A4 transcripts.

Taken together, these data suggest that the non-infectious transcripts are normally replicated after transfection, but cannot initiate a second cycle of viral multiplication. Thus it seems likely that mutations 1A1, 1A3 and 1A4 block one of the steps between RNA replication in a cell and the presence of newly replicated copies of the viral genome in neighboring cells. These steps include translation and processing of the viral polyprotein, virus assembly and release, attachment of virions to the cell receptor and uncoating of the viral RNA.

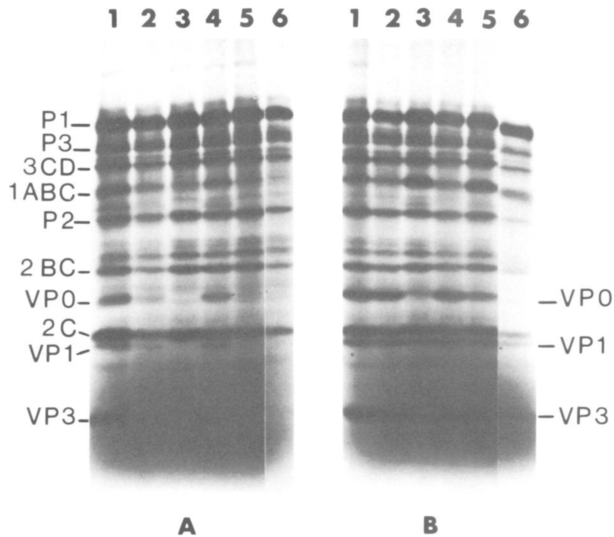

Fig. 5. *In vitro* translation and processing of mutated polyproteins. PV1 RNA (**lane 1**) or full-length transcripts prepared from pT7 PV1-51 (**lane 2**), or from p1A1 to p1A4 (**lanes 3–6**) were translated for 3 h at 30°C in a reticulocyte lysate supplemented with uninfected HeLa cell lysate (see Materials and methods). Translation products were then incubated for 1 h at 30°C with lysis buffer (**A**) or with PV1-infected HeLa cell lysate (**B**) and analyzed by SDS–PAGE. Poliovirus protein precursors P1(1ABCD), P2(2ABC) and P3(3ABCD) as well as some of their major cleavage products are indicated on the left. Viral capsid polypeptides are indicated on the right.

Effect of the mutations on translation and processing of the viral polyprotein

The effects of the mutations on translation of the various transcripts and the subsequent proteolytic processes of the polyprotein were studied *in vitro*. Translations were carried out in a reticulocyte lysate supplemented with an uninfected HeLa cell lysate. After addition of puromycin, the translation products were incubated further in the absence or in the presence of a poliovirus-infected HeLa cell lysate and analyzed by SDS–PAGE (see Materials and methods). In the absence of added poliovirus-infected cell lysate (Figure 5A), cleavage of the polyprotein into P1, P2 and P3 as well as processing of precursors P2 and P3 seemed to occur normally in the case of all transcripts. However, cleavage of precursor P1 (1ABCD) into VP0 (1AB), VP3 (1C) and VP1 (1D), while occurring normally with transcripts pT7 PV1-51 and p1A2, was very inefficient with transcript p1A3 and could not be detected in the case of transcripts p1A1 and p1A4. When a poliovirus-infected cell lysate was added to provide exogenous proteinase (Figure 5B), P1 cleavage was still incomplete in the case of the non-infectious transcripts. As indicated by the amount of VP1, the VP3–VP1 cleavage seemed to be roughly the same in the case of infectious and non-infectious transcripts. However, as judged by the relative intensities of the bands corresponding to VP3, VP0 and 1ABC (VP0–VP3), the VP0–VP3 cleavage was less efficient with the non-infectious transcripts from p1A1, p1A3 and p1A4 than with infectious transcripts. Since we were unfortunately unable to study the myristoylation of VP0 in the *in vitro* translation system, we cannot state with certainty that the imperfect cleavage of P1 is due to its lack of myristoylation. Nevertheless, we are confident that this is so, as those mutations that affect the myristoylation of VP0 *in vivo* are also those that hinder the proteolytic cleavage of P1 *in vitro*. These results therefore suggest that mutations Gly1 → Arg, Ala2 → Pro and Ser5 → Pro, which prevent the myristoylation of P1 and alter its cleavage *in vitro*, might, as a consequence, block viral assembly.

Discussion

Using site-directed mutagenesis, we have created four different mutations in the region of the poliovirus cDNA corresponding to the myristoylation signal of capsid protein VP4 (Met0 Gly1 Ala2 Gln3 Val4 Ser5 Ser6).

We have shown that the mutation of Ser5 to Thr still allows myristoylation of VP0, whereas the mutation of Gly1 to Arg completely prevents it. Mutations of Ala2 or Ser5 to Pro severely impede myristoylation, but the inhibition is not complete. Transcripts harboring mutation Ser5 to Thr were as infectious as wild-type transcripts, whereas the three other mutations rendered the transcripts non-infectious. However, a delayed production of virus was sometimes observed in the latter case. This seems to have resulted from reverse mutations occurring in the altered myristoylation sequence either during *in vitro* transcription or during RNA replication in the transfected cells. These revertants had recovered a functional myristoylation sequence and showed myristoylation of VP0. It is very unlikely that viral production was due to contamination, since the genome of all recovered viruses bore the same G → A mutation, located at nt 806, as the parental cDNA in plasmid pT7 PV1-51. In addition, some of the revertant viruses harbored another point mutation which was introduced during mutagenesis (a C at the third position of the Gly1 codon for v1A1, and an A at the third position of the Val4 codon for all v1A3 viruses; see Table II).

We can thus define the following rules for the myristoylation signal of poliovirus VP4. Position 1 is always Gly, in agreement with other reports (Towler *et al.*, 1988b and references therein). Position 2 can accommodate Thr, Ala, Ser and Leu, whereas Pro, Arg and His seem to be excluded. At position 5, Thr, Ser and Ala are accepted whereas Pro, Gln, Leu and Arg seem to be excluded. As reversions from a Pro codon can only give six different codons through a point mutation, it is conceivable that some other amino acids may be found at positions 2 or 5. Indeed, among the known myristoylation signal sequences, Asn, Gln, Ala, Ser, Gly, Cys or Val residues have been found at position 2 and Ala, Ser, Thr or Gly residues at position 5 (Towler *et al.*, 1988b). Furthermore, using octapeptides as substrates for purified yeast or rat liver NMT, Towler *et al.* (1987b, 1988a) demonstrated that Cys and Asn residues are also allowed at position 5, whereas Pro, charged residues or residues with bulky hydrophobic side chains at positions 2 or 5 result in peptides that are either inactive or very poor substrates (reviewed in Towler *et al.*, 1988b). Overall, our results are in good agreement with these observations. In particular, we demonstrate here that a Thr2 residue is allowed. However, while an octapeptide derived from the cAMP-dependent protein kinase with a Leu2 substitution was shown to be a poor substrate and even an inhibitor of yeast NMT (Towler *et al.*, 1987a), Leu2 is accommodated in the context of the myristoylation signal of poliovirus VP4. Furthermore, in this context, Pro2 and Pro5 substitutions seem to allow partial myristoylation of VP0.

The mutated, myristoylation-negative transcripts were devoid of infectivity. We found that they could replicate

normally in the transfected host cell, but failed to initiate a second cycle of viral multiplication. Given the N-terminal position of myristate in the P1 capsid precursor protein, and knowing its location in the three-dimensional structure of the poliovirion, the different steps which could involve the myristate moiety are: (i) viral adsorption and decapsidation, (ii) proteolytic cleavage of precursor P1 and (iii) viral assembly, either at the stage of assembly of 5S protomers into 14S pentamers or at that of 14S pentamers into virions.

The possible role of myristoylation of VP4 in viral adsorption and/or decapsidation is supported by the observation that viral particles, once adsorbed on their cellular receptors, are subject to a conformational change which is accompanied by the externalization of VP4 (Lonberg-Holm and Korant, 1972; Lonberg-Holm *et al.*, 1975). Willingmann *et al.* (1989) recently reported that intact, infectious viral particles could be recovered from inside the cell after receptor-mediated endocytosis, suggesting that myristate does not play a role in viral adsorption. The question of whether myristoylation of VP4 is required for viral decapsidation has so far not been addressed.

The lack of *in vivo* myristoylation of VP0 could be correlated with the lack of cleavage of P1 in an *in vitro* translation system. This lack of P1 cleavage *in vitro* could be partially overcome by addition of exogenous proteinase. The cleavage between VP3 and VP1 was then almost complete, but the resulting precursor protein 1ABC (VP0−VP1) remained mostly resistant to further processing. Preferential processing of P1 at the VP3−VP1 cleavage site has been reported *in vitro* in the presence of excess purified 3C proteinase (Nicklin *et al.*, 1988) and this cleavage occurs before the VP0−VP3 cleavage in the case of EMCV (Shih and Shih, 1981; Jackson, 1986). Proper folding of P1 is known to be crucial for its processing *in vitro* (Arnold *et al.*, 1987; Ypma-Wong and Semler, 1987; Ypma-Wong *et al.*, 1988). In particular, it has been shown that a P1 precursor truncated of most of the VP4 sequence is very inefficiently processed *in vitro* (Nicklin *et al.*, 1987). It is therefore tempting to speculate that the myristate moiety might act as the initiator for proper folding of P1. Alternatively, the proteolytic cleavage of P1 could necessitate its anchoring to membranes, and this could be achieved via the myristate moiety. Several myristoylated proteins were shown to be membrane-associated (Magee and Courtneidge, 1985; Pellman *et al.*, 1985) and Rhee and Hunter (1987) demonstrated that myristoylation of the Mason−Pfizer monkey virus gag-polyprotein precursor is required for its association with the plasma membrane and its proteolytic cleavage *in vivo*.

Poliovirion assembly is thought to occur in the vicinity of the site of RNA replication which is located on membranous structures (Girard and Baltimore, 1967; Caliguiri and Compans, 1973). It is possible that the myristate moiety of VP0 plays a role there, by maintaining a high concentration of protomers close to the replication site. In agreement with this hypothesis, Rein *et al.* (1986) have shown that myristoylation of $Pr65^{gag}$ is required for virus particle formation at the plasma membrane in the case of Moloney murine leukemia virus. More simply, the myristate moiety of VP0 could be involved in the association of 5S protomers to build 14S pentamers. The 5-fold-related myristates constitute a hydrophobic cluster of 65 methyl groups which could account for the stability of the β-tube, and hence of the whole pentamer. Experiments are in progress to investigate this possibility by analyzing the presence of 14S pentamers or 74S procapsids in the transfected cells.

Materials and methods

Bacterial strains and plasmids

Bacterial strains *Escherichia coli* 1106 (803 r_k^- m_k^-) (Murray *et al.*, 1976) and HB101 (Boyer and Roulland-Dussoix, 1969) were respectively used for propagation of plasmids and transformation following mutagenesis.

Plasmid pT7 PV1-51 was a derivative of pT7 PV1-5 (van der Werf *et al.*, 1986) in which the *Nhe*I−*Bgl*II fragment from nt 2470−5601 of the poliovirus type 1 (Mahoney) cDNA has been replaced by the equivalent cDNA fragment from plasmid pPV1-1515 (Girard *et al.*, 1985). In addition, it carries a (G → A) mutation at position 806 (our unpublished observation). Plasmid pT7 PV1-52 was derived from pT7 PV1-51 by destroying the *Aat*II and *Bam*HI sites of the pBR322 sequence. Plasmid pSW5004, which contains only the first 1118 nt of poliovirus cDNA, was derived from pT7 PV1-5 by digestion with *Aat*II and recircularization with T4 DNA ligase; in addition, it lacks the *Bam*HI site at position 375 of the pBR322 sequence. Plasmid pSW3002 contains the poliovirus cDNA sequence from nt 4830−1 following promoter φ10 of bacteriophage T7 and inserted between nt 375 and 2297 of pBR322.

Recombinant DNA procedures were essentially as described (Maniatis *et al.*, 1982). Restriction and modification enzymes (Boehringer Mannheim, BRL or Biolabs) were used according to the manufacturer's instructions.

Oligonucleotide site-directed mutagenesis

Plasmid pSW5004 was, on the one hand, cleaved at the *Nde*I site and dephosporylated with calf intestinal phosphatase (CIP), and, on the other hand, hydrolyzed with *Bam*HI alone or with both *Aat*II and *Bam*HI. Fragments *Bam*HI−*Bam*HI (450 bp) and *Aat*II−*Bam*HI (4160 bp) were isolated in a low gelling temperature agarose gel. The linearized plasmid and the latter two fragments were mixed in equimolar ratios (0.05 pmol) with a molar excess of the phosphorylated synthetic oligonucleotide (20 pmol; Igolen, Institute Pasteur). This mixture was denatured for 3 min at 90°C, cooled slowly to allow annealing, then incubated overnight at 15°C with 4 U of Klenow enzyme and 4 U of T4 DNA ligase as described by Morinaga *et al.* (1984). HB101 cells were transformed and the resulting clones screened by *in situ* colony hybridization using, as a probe, the 5′ ^{32}P-labeled oligonucleotide involved in the mutagenesis. Hybridization at 42°C was followed by step-by-step washings at increasing temperatures (Wallace *et al.*, 1979). Mutant plasmids, pDMi, which still hybridized at the highest temperature, were checked for the presence of a new restriction site, and their DNA sequence was determined in the region of the mutation (Zagursky *et al.*, 1985).

In vitro transcription and purification of transcripts

Unless otherwise stated, purified plasmids were linearized with *Eco*RI and transcribed for 30 min at 37°C with T7 RNA polymerase (Genofit) as previously described (van der Werf *et al.*, 1986). The concentration of the transcripts was estimated by agarose gel electrophoresis. For *in vitro* translation experiments, transcription mixtures were extracted once with phenol/chloroform (1:1) and once with chloroform, then precipitated once with 2 M LiCl and twice with ethanol. The concentration of the recovered transcripts was determined by reading of the OD_{260}. For the preparation of a ^{32}P-labeled riboprobe complementary to nt 3417−4830 of the viral RNA, plasmid pSW3002 was hydrolyzed with *Pst*I and transcribed with T7 RNA polymerase in the presence of 0.04 mM UTP and 1 μCi/μl of [α-^{32}P]UTP (>400 Ci/mmol, Amersham). The transcription mixture was then treated for 10 min at 37°C with RQ1 DNase (1 U/μg DNA, Promega) and labeled RNAs were purified by chromatography on Sephadex G75 (Pharmacia) columns.

Transfections

Confluent monolayers of HeLa or VERO (VC-10) cells were transfected with appropriate dilutions of the transcription mixtures in the presence of DEAE−dextran as previously described (van der Werf *et al.*, 1986). Transfected cells were overlaid with Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum (FCS) or, in the case of titrations, with DMEM, 2% FCS containing 50 mM $MgCl_2$ and 0.9% agar noble, and incubated at 37°C, unless otherwise stated.

Extraction of cytoplasmic RNAs

HeLa cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated for 10 min on ice in TEN (10 mM Tris−HCl, pH 7.4, 1 mM EDTA, 0.14 M NaCl), 0.5% NP-40. Following removal

of the nuclei, the cytopasmic extracts were extracted with phenol/chloroform (1:1), then with chloroform and the RNAs were precipitated with ethanol. RNA concentration was determined by reading of the OD_{260}.

Sequencing of viral RNA

HeLa cells were infected at a multiplicity of infection (m.o.i.) of 40 p.f.u./cell. Cytoplasmic RNAs (10 μg), purified at 5 h post-infection (see above), were annealed to 0.1 μg of 5′ ^{32}P-labeled oligonucleotide primer complementary to nt 847–862 of the viral RNA and sequencing reactions were carried out using AMV reverse transcriptase (Boehringer) as described (Geliebter *et al.*, 1986).

Dot-blot hybridization of viral RNA

Cytoplasmic RNAs (1 μg) from transfected HeLa cells were denatured for 15 min at 65°C in 6 × SSPE (0.9 M NaCl, 0.06 M NaH_2PO_4, 6 mM EDTA), 6% formaldehyde, then filtered through a nylon filter (Hybond N, Amersham) using a dot-blot apparatus (Manifold, BRL) and finally bound to the filter by a 3 min UV irradiation. Prehybridization was for 3 h at 52°C in hybridization buffer [50% formamide, 5 × SSPE, 0.1% SDS, 5 × Denhardt's]. Hybridization was performed overnight at 52°C with 10^8 c.p.m. of ^{32}P-labeled RNA probe in 50 ml of hybridization buffer. The filter was then washed four times for 10 min at room temperature in 2 × SSPE, 0.1% SDS, twice for 30 min at 52°C in 1 × SSPE, 0.1% SDS, 1 h at 60°C in 0.5 × SSPE, 0.1% SDS and 1 h at 67°C in 0.1 × SSPE, 0.1% SDS.

In vivo labeling of viral proteins and immunoprecipitations

Confluent HeLa cell monolayers on 35 mm plates were infected at an m.o.i. of 40 p.f.u./cell or transfected as described above. Cells were labeled 3 h later either with [^{35}S]methionine (30 μCi/ml; >1000 Ci/mmol; Amersham), after a 30 min incubation in DMEM minus methionine, or with [9,10(*n*)-^{3}H]myristic acid (200 μCi/ml; 40–60 Ci/mmol, Amersham). At 7 h post-infection or 7.5 h post-transfection, cells were washed three times with ice-cold PBS and lysed in 40 μl TEN, 0.5% NP-40 (see above). Twenty microliters of 3 × Laemmli buffer (Laemmli, 1970) were added to the cytoplasmic extracts and 10–30 μl of the samples were immunoprecipitated with an αVP2 or an αC polyclonal rabbit serum essentially as described (Emini *et al.*, 1985). The immune precipitates were analyzed by electrophoresis on a 15% SDS–polyacrylamide gel (Laemmli, 1970). The gel was fixed, treated with Amplify (Amersham), dried and subjected to autoradiography at −70°C in the presence of an intensifying screen.

In vitro translation

Reticulocyte lysates, prepared as described by Jackson and Hunt (1983), were treated with nuclease and used essentially as in Bénicourt *et al.* (1978). The reaction mixtures also contained 2 μl of uninfected HeLa cell lysate and 0.24 μg of mRNA. It was incubated for 3 h at 30°C and the reaction was stopped by addition of 0.2 mM puromycin. The samples were then incubated for 1 h at 30°C with 0.2 μg RNase A and 6 μl either of poliovirus-infected HeLa cell lysate or of lysis buffer (10 mM KCl, 1.3 mM Mg acetate, 2.5 mM DTT, 10 mM Hepes KOH, pH 7.4) and finally analyzed by electrophoresis on a 12.5% SDS–polyacrylamide gel (Laemmli, 1970). The gel was fixed and subjected to fluorography (Laskey and Mills, 1975) before being autoradiographed. The uninfected and PV1-infected HeLa cell lysates were prepared essentially as described (Brown and Ehrenfeld, 1979; Semler *et al.*, 1981) and nuclease treated as the reticulocyte lysate.

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