Analysis of the functional significance of amino acid residues in the putative NTP-binding pattern of the poliovirus 2C protein

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The amino acid sequence of the poliovirus 2C protein contains two highly conserved stretches, GSPGTGKS and MDD, which correspond to the consensus 'A' and 'B' motifs (GXXXXGKS/T and DD/E, respectively) found in nucleoside triphosphate-binding proteins. To assess the functional importance of these amino acid sequences, we changed conserved and non-conserved amino acids. The replacement of the non-conserved Thr residue with Ser or Ala did not markedly change the virus phenotype. Similarly, replacement of the non-conserved Pro residue by Ala did not abolish virus viability, but changes of this residue to Thr or Asn were not tolerated. No viable mutant could be isolated after transfection of cultured cells with transcripts mutated at the conserved Lys, Ser, or Asp residues. However, true revertants were selected from Arg and Ser mutants, from Glu and Gly mutants, and from Ala mutants. Thr mutants not only gave rise to true revertants, but also to two independent isolates of a suppressor mutant, Asn-Tyr. All the lethal mutations resulted in severe inhibition of viral RNA synthesis in vivo, although no translational deficiency was detected in a cell-free system. This is the first direct evidence for the functional significance of the nucleoside triphosphate-binding pattern in the poliovirus 2C protein.

Introduction

Poliovirus, a member of the picornavirus family, contains a positive-sense ssRNA genome of 7440 nucleotides. The viral genome encodes a single polyprotein which is proteolytically cleaved to produce the mature viral proteins. The structural proteins, which form the viral capsid, are located in the amino-terminal P1 region of the polyprotein, whereas the viral polymerase (3D), a protease (3C) and the genome-linked small polypeptide (VPg) are located in the carboxy-terminal P3 region (Pallansch et al., 1984). The central region of the viral polyprotein, P2, encompasses three non-structural polypeptides, 2A, 2B and 2C. Protein 2A appears to be the protease responsible for cleavage of the polyprotein at the P1-P2 junction (Toyoda et al., 1986) and is involved in the inactivation of the cellular translation initiation factor eIF-4F (Kräusslich et al., 1987). The roles of proteins 2B and 2C remain much more obscure. Their importance for viral RNA synthesis can be inferred from the results of their mutagenesis (Bernstein et al., 1986; Johnson & Sarnow, 1991; Li & Baltimore, 1988).

Poliovirus mutants containing short insertions within the C-terminal domain of the 2C protein have been shown to have temperature-sensitive defects in viral RNA synthesis, whereas insertions within the middle region of this polypeptide abolish virus replication (Li & Baltimore, 1988). Furthermore, all the precisely characterized mutations conferring resistance to, or dependence on, guanidine hydrochloride map to the same middle domain of the 2C protein (Pincus & Wimmer, 1986; Pincus et al., 1986, 1987; Baltera & Tershak, 1989). Although the precise mode of action of this drug remains unknown, its major effect appears to be the blockage of viral RNA synthesis (Caliguiri & Tamm, 1968, 1973; Nobel & Levintow, 1970).

Sequence comparisons reveal strong conservation of the middle part of the 2C protein sequence among picornaviruses (Argos et al., 1984) and even between picorna-, como-, nepo- and caliciviruses (Franssen et al., 1984; Gorbalenya et al., 1990; Neil, 1990). Within this region, the sequence elements corresponding to the
conserved motifs found in numerous nucleoside triphosphate-binding (NTP-binding) proteins are present (Gorbaleyna et al., 1985). This NTP-binding pattern comprises the so called ‘A’ motif, a Gly-rich loop preceded by a stretch of three and five hydrophobic residues and followed by a conserved Gly–Lys–Ser(Thr) sequence (usually represented as the consensus sequence GXXXXGKS/T), and the ‘B’ motif, an Asp residue usually followed by an Asp or Glu residue and preceded by a stretch of three to five hydrophobic residues (Walker et al., 1982; Gorbaleyna & Koonin, 1989). If present in a protein in an ordered fashion, these motifs are highly indicative of associated nucleotide-binding activity (Dever et al., 1987; Gorbaleyna & Koonin, 1989). In the most extensively studied nucleotide-binding proteins, the human ras oncogene protein and elongation factor Tu of *Escherichia coli*, these two consensus motifs have been shown to constitute the phosphoryl/Mg-binding sequences (Jurnak, 1985; De Vos et al., 1988).

Extensive computer analysis of proteins that contain the NTP-binding pattern has demonstrated that picornavirus 2C proteins and their homologues in other families of RNA viruses can be assigned to the superfamily that contains the ATP-binding proteins of several DNA viruses with small genomes (Gorbaleyna et al., 1990). Among these proteins are papovavirus T antigen and parvovirus NS1 protein, which possess DNA-dependent ATPase and helicase activities (Stahl et al., 1986, Im & Muzychka, 1990). Based on these facts, it has been suggested that picornavirus 2C proteins may function as RNA helicases (Gorbaleyna et al., 1990). Recently, such nucleic acid-stimulated ATPase and RNA unwinding activities have been identified for plum pox potyvirus CI protein, another protein which contains the NTP-binding pattern mentioned above (Lain et al., 1990, 1991).

As part of a functional characterization of the poliovirus 2C protein, we investigated the significance of individual amino acid residues within the nucleotide-binding A and B motifs. To this end, we used genetic manipulation of poliovirus cDNA clones which produce infectious virus upon transfection of primate cells. The results reported here demonstrate that when conserved amino acids within the A or B motif of the NTP-binding pattern are replaced, even by similar residues, virus replication can not be detected. In contrast, similar mutations which changed nearby amino acids within the A motif that are non-conserved in terms of nucleotide-binding pattern did not prevent virus replication and viable viruses could be isolated. Our data show that conservation of the A and B motifs of the NTP-binding pattern within picornavirus 2C proteins therefore may be functionally significant.

### Methods

**Bacterial strains and plasmids.** Bacterial strains *E. coli* 1305 and TG1, respectively, were used for the propagation of plasmids and bacteriophages. The construction of transcription vector pT7-PV1-52, harbouring a full-length cDNA of poliovirus type 1 (Mahoney strain) has been described previously (Marc et al., 1989). Plasmid pKK61, which contains poliovirus cDNA between nucleotides 2470 and 5824 was constructed by insertion into pBR322 of the 3.3 kb *NheI–NarI* fragment from pK7K (Kean et al., 1986). For site-directed mutagenesis, two subclones were constructed containing poliovirus cDNA from nucleotides 3412 to 4600 (M13-NT-2C-A) or from nucleotides 4600 to 5601 (M13-NT-2C-B) inserted into bacteriophage M13mp19. Recombinant DNA procedures used were essentially as described (Sambrook et al., 1989). Restriction and modification enzymes (Boehringer Mannheim, BRL or Biolabs) were used as directed by the manufacturers.

**Oligonucleotide-directed site-specific mutagenesis and reconstruction of full-length mutants.** Oligonucleotide-directed *in vitro* mutagenesis reactions were carried out using the Amersham mutagenesis system (version 2) as described by the manufacturers. Briefly, phosphorylated oligonucleotides were annealed to ssDNA templates, and mutant DNA strands were synthesized and ligated in the presence of a dNTP mixture which contained dCTPdS, the Klenow fragment of DNA polymerase I and T4 DNA ligase. The remaining non-mutant ssDNA was removed by filtration through a nitrocellulose membrane. The non-mutated strand of dsDNA was nicked using *NciI*, and partially digested using exonuclease III. After repolymerization and ligation of the gapped DNA, competent *E. coli* TG1 cells were transformed to give mutant bacteriophage progeny.

The sequences of the oligonucleotides used for mutagenesis (Igolen, Pasteur Institute) are shown in Fig. 1. Each group of oligonucleotides contains a mixture of bases at certain positions, allowing different mutations of a single codon to be obtained during a single mutagenesis reaction. Bacteriophage M13-NT-2C-A or M13-NT-2C-B ssDNA was used for the mutagenesis of A or B motif amino acids, respectively. The bacteriophage clones obtained were screened by sequencing the appropriate region of the ssDNA using the T7 DNA polymerase sequencing kit (Pharmacia LKB). The sequence of each mutated clone was determined over the entire region used for further reconstructions.

Mutations were inserted into a full-length poliovirus cDNA by a two-step cloning procedure. In the first step, the 1.2 kb PstI-BamHI fragment of pKK61 was replaced by that of different dsM13-NT-2C-A mutants (positions 3412 to 4600 of the poliovirus cDNA), or the 0.7 kb BamHI–BglII fragment of pKK61 was replaced by that of dsM13-NT-2C-B mutants (positions 4600 to 5318 of the poliovirus cDNA). The sequence of mutant plasmid dsDNA was verified over the mutagenesis and insertion sites. In the second step, the mutations were introduced into plasmid pT7-PV1-52 by substitution of the 3.1 kb *Nhel–BglII* fragment (positions 2470 to 5601 of the poliovirus cDNA). The sequence of each full-length cDNA construct was verified over the mutagenesis site. In addition, detailed restriction mapping of these plasmids was undertaken to ensure that no major rearrangements or deletions had occurred. The plasmids generated were named according to the nomenclature proposed by Bernstein et al. (1986), pG1-2C-S150T, pG1-2C-S136A, pG1-2C-K135R, pG1-2C-K135S, pG1-2C-T123S, pG1-2C-T123R, pG1-2C-P131A, pG1-2C-P131T, pG1-2C-P131N, pG1-2C-D177E and pG1-2C-D177G (Table 1), although pSer138Thr, pSer138Ala, etc. will be used in the text.

**RNA transcription and transfection.** RNA was synthesized from pT7-PV1-52 or its mutant derivatives (Table 1) in 50 to 150 μl reaction mixtures containing 20 mM-KH2PO4, 5 mM-HPO4, pH 7.5, 8 mM-MgCl2, 4 mM-spermidine, 10 mM-DTT, 200 μM each NTP, 1 unit/μl RNasin.
Fig. 1. Site-specific mutagenesis of the 2C protein putative nucleotide-binding pattern. (a) Diversity of selected positions of putative NTP-binding patterns in 2C-like proteins encoded by genomes of the viruses of the picornavirus-like supergroup, and substitutions introduced at these positions. The sequences of the A and B motifs within the 2C protein of poliovirus are shown. For other viruses, only the residues corresponding to those mutated in this study are shown, according to the alignment of protein sequences published previously (Gorbalenya et al., 1981). Alternations of the residues in the 2C protein like supergroup are shown in italics. The sequences of the synthetic oligonucleotides used during mutagenesis are shown below each oligonucleotide sequence. Oligonucleotides 3 and 4 introduced t A s a r T E

(b) A motif

(c) B motif

DNA and cDNA sequencing. DNA was prepared from virus-infected cells for direct sequencing and for cDNA synthesis. HeLa cell monolayers were infected at a multiplicity of 20, and RNA was extracted from the infected cells after 6 to 8 h, by cell lysis with NP40 (Marc et al., 1989). Direct sequencing of viral RNA was performed as described (Fichot & Girard, 1990).

Analysis of viral RNA replication. HeLa cell monolayers were transfected with full-length wild-type or mutant RNA transcripts as described above. At various times after transfection the cytoplasmic RNA were as described previously (Marc et al., 1989). Alternative, RNA pulse labelling of 30 min from 5 to 5.5 h post-infection was done. In this case, dactinomycin (2.5 μg/ml) was added only 15 min prior to the addition of [3H]uridine (Agol et al., 1984).
Table 1. Mutants obtained after mutagenesis of the 2C protein putative NTP-binding site

<table>
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<tr>
<th>Plasmid</th>
<th>2C protein A motif†‡</th>
<th>Transfection efficiency†‡ (log10 p.f.u./µg)</th>
<th>C.p.e. production*</th>
<th>Number of virus plaques sequenced¶</th>
<th>2C protein A motif**</th>
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<td>10/10</td>
<td>&lt;24</td>
<td>5</td>
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<td>28-35</td>
<td>6</td>
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<td>6††</td>
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<td>2/10</td>
<td>&gt;52</td>
<td>1††</td>
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</table>

* C.p.e. after transfection at 37 °C in liquid medium.
† Sequences encoded by plasmid; the mutation is underlined.
‡ Estimated after transfection at 37 °C under a semi-solid medium.
§ Ten plates were scored for c.p.e. for each plasmid, and the presence of infective virus was verified by titration.
‖ Time of appearance of 100% c.p.e. at 37 °C.
¶ Plaques were isolated after transfection at 37 °C under semi-solid agar.
** The sequence was determined by sequencing viral RNA.
†† NR. No result.
‡‡ In these cases, RNA from all the plaques isolated was sequenced.
§§ An additional mutation was found in two of the six plaque isolates sequenced.

Results

Site-directed mutagenesis of the 2C protein nucleotide-binding pattern

In an attempt to determine the functional importance of the nucleotide-binding pattern present in the sequence of the poliovirus 2C protein, point mutations were introduced into this region of the viral cDNA. As targets for mutagenesis, the following amino acid positions were chosen: highly conserved residues Lys135, Ser136 and Asp177, and non-conserved residues Pro131 and Thr133 (Fig. 1a). Ser136 (UCU) was changed to Thr (ACU) or Ala (GCU), Lys135 (AAA) to Arg (AGA) or Ser (AGC), and Asp177 (GAC) to either Glu (GAG) or Gly (GGG). Similarly, Pro131 was changed to Ala, Thr or Asn, and Thr133 to either Ser or Ala (Fig. 1b, c).

The residues introduced to replace those occurring naturally in poliovirus can be classified into four groups. Conserved residues were changed (i) to similar residues found at the equivalent positions of 2C-like proteins of picornavirus-like viruses (mutations Ser136Ala, Asp177Gly, Lys135Arg and Lys135Ser). Non-conserved residues situated within the A motif were changed (i) to residues found at the equivalent positions in 2C proteins of picornaviruses other than poliovirus (Pro131Ala, Thr133Ser and Thr133Ala) or (ii) to residues not observed in picornaviruses, but which occur in other NTP-binding proteins (Pro131Thr and Pro131Asn).

Chosen mutations were introduced into poliovirus cDNA by oligonucleotide-directed site-specific mutagenesis (see Methods). The synthetic oligonucleotides used for mutagenesis were redundant at certain positions so that during a single mutagenesis reaction different mutations of the same codon could be introduced (Fig. 1b, c). Mutants were selected by direct sequencing of the appropriate region of bacteriophage clones. The DNA of each mutant clone was then sequenced over the whole region used for further reconstruction (nucleotides 3417 to 4600 for A motif mutants or nucleotides 4600 to 5318 for B motif mutants) to verify that no mutations other than those intended had been introduced during mutagenesis. The mutations were next reintroduced into a plasmid carrying the infectious full-length poliovirus cDNA downstream of the T7 φ10 promoter (see Methods). The plasmids generated will be called

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pSer\textsuperscript{136}Thr, pSer\textsuperscript{136}Ala, etc., and the viruses or viral RNAs carrying mutations Ser\textsuperscript{136}→Thr, Ser\textsuperscript{136}→Ala etc. The presence of the mutations in these plasmids was confirmed by sequencing. As we did not sequence the entire poliovirus cDNA, spontaneous mutations occurring at other sites during the recloning procedure could not be entirely excluded. Therefore, for mutants Ser\textsuperscript{136}→Thr and Ser\textsuperscript{136}→Ala, two independent full-length cDNA clones were reconstructed using two independent mutant subclones, and experiments were carried out using both cDNA molecules.

Viability of the 2C mutants

To study the effects of the mutations on virus viability, wild-type and mutant plasmids (Table 1) were linearized using EcoRI and transcribed in vitro using T7 RNA polymerase. The specific infectivity of the genomic transcripts, or the appearance of c.p.e. was determined following transfection of HeLa cells at 35 °C and 37 °C. Viral RNA was prepared for sequence analysis either from transfection supernatants, or from individual plaques isolated after transfection (see Methods). No difference in results was observed upon transfection of HeLa cells at 35 °C and 37 °C. Thus only the data obtained at 37 °C will be discussed below.

The mutant transcripts could be divided into three groups with regard to their ability to produce infectious progeny. The transcripts from pThr\textsuperscript{133}Ser, pThr\textsuperscript{133}Ala and pPro\textsuperscript{131}Ala exhibited a specific infectivity of the same order of magnitude as the wild-type transcript (Table 1). After transfection with 0.1 pg of RNA per cell, c.p.e. was visible at about the same time for wild-type virus and for mutants Thr\textsuperscript{133}→Ser and Thr\textsuperscript{133}→Ala, or with a short delay for Pro\textsuperscript{131}→Ala (Table 1). The viral RNA sequence obtained from mutant virus stocks or individual plaques isolated after transfection revealed no replacements other than the engineered mutations, at least within positions 4430 to 4590 (data not shown). The phenotypic properties of these mutants are described below.

The transcript from pPro\textsuperscript{131}Thr belonged to a second group. Its specific infectivity was 10\textsuperscript{2} to 10\textsuperscript{3}-fold lower than that of wild-type virus (< 10\textsuperscript{3} p.f.u./μg). Sequence analysis of the RNA from virus produced from either six single plaques isolated immediately after transfection or four ministocks prepared after c.p.e. development showed that these viruses were primary site revertants. They encoded the wild-type Pro at position 131, but had retained a silent mutation at position 4507 which was present in the mutant but not in the wild-type RNA (see Fig. 1b).

Finally, the transcripts from pSer\textsuperscript{136}Thr, pSer\textsuperscript{136}Ala, pLys\textsuperscript{135}Arg, pLys\textsuperscript{135}Ser, pAsp\textsuperscript{177}Glu, pAsp\textsuperscript{177}Gly and pPro\textsuperscript{131}Asn were considered to be non-infectious in titration experiments (specific infectivity < 10 p.f.u./μg). However, virus was occasionally recovered from plates transfected with the RNA transcripts from all of these plasmids, except pPro\textsuperscript{131}Asn. This was a late (3 to 5 days post-transfection) sporadic event of variable frequency (see Table 1).

Individual virus ministocks were prepared from each plate in which c.p.e. was observed (either directly or after plaque purification), and the sequence of the viral RNA in the region encoding the putative nucleotide-binding pattern of the 2C protein was determined. In addition, material from individual virus plaques isolated after transfection under semi-solid agar was used to produce cDNA corresponding to the 2C protein coding region, and the cDNA fragments obtained were sequenced directly. In the case of pLys\textsuperscript{135}Arg, pLys\textsuperscript{135}Ser, pSer\textsuperscript{136}Ala, pAsp\textsuperscript{177}Glu and pAsp\textsuperscript{177}Gly, both types of experiment showed that the RNA of the virus progeny encoded a 2C protein with a wild-type amino acid sequence. Thus, the viruses obtained were characterized as primary site revertants. pSer\textsuperscript{136}Thr was slightly different from the other non-infectious transcripts. Most samples consisted of virus that had undergone spontaneous mutation restoring the wild-type amino acid sequence of the 2C protein (either A to T at nucleotide 4529, or C to G at nucleotide 4530). However, two virus stocks produced from plaques derived from two independent clones of pSer\textsuperscript{136}Thr had retained the ACT codon responsible for the Ser\textsuperscript{136}→Thr change. In both cases there was a second mutation of A to T at nucleotide 4541 which introduced a change of Asn\textsuperscript{140}→Tyr within the 2C protein. The properties of this putative suppressor mutation will be described elsewhere (unpublished results).

It seems unlikely that the sporadic virus production observed after transfection of non-infectious transcripts can be attributed to contamination with wild-type virus. Unequivocal evidence against this possibility was provided in the case of transcripts from pSer\textsuperscript{136}Thr and pAsp\textsuperscript{177}Glu. In these cases reversion to the wild-type amino acid sequence was due not only to the restoration of the original codon, but also to the acquisition of synonymous codons. The absence of this phenomenon for the other mutants can be explained by the restrictions imposed by the genetic code. Thus, the absence of the engineered mutations in the genomes of the viruses recovered could most probably be explained by spontaneous point mutations.

Effect of the mutations on the replication of viral RNA

To investigate at which step virus multiplication was blocked in the case of lethal mutations, we analysed the
ability of mutated transcripts to replicate after transfection. To this end, the accumulation of virus-specific RNA in cells transfected with the mutant transcripts was investigated by slot-blot hybridization (Fig. 2). Such an analysis has been used previously to detect the replication of RNA transcripts bearing lethal mutations that restrict viral RNA replication to a single cycle (Marc et al., 1989; Reuer et al., 1990). In addition, severely defective RNA replication can be detected by such a method (our unpublished data on 3CPro mutants). However, this method should be considered as only semi-quantitative. Replication of the wild-type full-length transcript of pT7-PV1-52 was first detectable 5 to 7 h after transfection, depending on the experiment (see Fig. 2). Replication of the Thr133→Ser and Thr133→Ala mutants was detectable at the same time as that of wild-type virus (Fig. 2). In agreement with the later appearance of c.p.e., the replication of the third viable mutant Pro131→Ala was detected later (15 to 24 h after transfection) (Fig. 2 and data not shown). For Pro131→Thr, a replication signal was not detectable until 24 h after transfection. Non-infectious transcripts (specific infectivity < 10 p.f.u./μg) gave no detectable viral RNA replication signal even as late as 24 h after transfection. However, 35 h after transfection, hybridization signals were often observed for transcripts from pSer136Thr, and occasionally for the other transcripts, in correlation with the sporadic c.p.e. observed for these mutants and resulting from the emergence of revertants.

To ascertain that RNA replication could only be detected once reversion had occurred in the case of non-viable mutant transcripts, we determined the sequence of the virus-specific RNA in the earliest samples of cytoplasmic extracts for which replication was detectable. In all cases, the appearance of a detectable replication signal correlated with the restoration of the wild-type amino acid sequence (data not shown).

To ascertain that the absence of detectable RNA accumulation prior to the emergence of revertants was related directly to replication defects and was not the result of translation deficiencies, the in vitro translation of transcripts in rabbit reticulocyte lysates supplemented with an uninfected HeLa cell extract was investigated. The template activity of all mutant RNA transcripts and the pattern of proteolytic processing of products were indistinguishable from those of wild-type RNA (data not shown). These data showed that the lethal mutations do not change the translation and proteolytic processing of the polyprotein encoded by these RNAs, at least in the in vitro system used.

**Phenotypic characterization of viable mutants**

The effects of the viable alterations within the 2C protein, Thr133→Ser, Thr133→Ala and Pro131→Ala, were
characterized in terms of plaque phenotype and viral RNA synthesis. The replacement of Thr$^{133}$ by Ser resulted in no apparent phenotypic alteration. On the other hand, a change of the same amino acid to alanine was accompanied by a small plaque phenotype at all temperatures tested (Fig. 3). A more complex situation was seen for virus stocks produced after transfection with RNA transcripts encoding the Pro$^{131}$→Ala change. These virus stocks always produced plaques of variable size, especially if the plaques were stained relatively early after infection (Fig. 3). Attempts to obtain a homogeneous small plaque population of Pro$^{131}$→Ala by cloning were unsuccessful. When virus isolated from small plaques was amplified by a single passage in cell culture, the small plaque phenotype was never retained. However, these stocks always conserves the expected CCC→GCC mutation at position 4514 (Pro→Ala) (data not shown). Thus, the possibility that additional small plaque type-suppressing mutation(s) had been acquired elsewhere could not be excluded.

We next examined viral RNA synthesis by measuring [$^{3}$H]uridine incorporation in mutant- and wild-type-infected cells at 37 °C and 39 °C in the presence of dactinomycin. This drug prevents the synthesis of host cell RNA, but does not inhibit viral RNA synthesis in cells infected with most poliovirus strains. Two criteria were considered, the maximum level of synthesis (expressed as a percentage of wild-type synthesis) and the time post-infection at which this maximum was reached (Table 2). Wild-type virus gave a maximum incorporation at 5 to 6 h after infection at 37 °C and at about 7 h after infection at 39 °C, the same level of incorporation being observed at both temperatures. The Thr$^{133}$→Ser mutant behaved similarly to wild-type virus at 37 °C, but showed decreased RNA synthesis at 39 °C. The Thr$^{133}$→Ala mutant demonstrated decreased RNA synthesis at 37 °C as well as at 39 °C. This correlates with the small plaque phenotype of this virus (Fig. 3). The Pro$^{131}$→Ala mutant was clearly deficient in RNA synthesis. At 37 °C, only 46% of the wild-type level of

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Fig. 3. Plaque phenotypes of Thr$^{133}$→Ser, Thr$^{133}$→Ala and Pro$^{131}$→Ala mutants. HeLa cell monolayers were infected with wild-type or mutant virus stocks and incubated at 35 °C, 37 °C or 39 °C (columns 1 to 3). Plaques were stained with crystal violet after incubation for approximately 48 h (a) or 68 h (b).
RNA synthesis was observed, and this was reached 10 h after infection. At 39 °C, RNA synthesis reached no more than 16% of that of wild-type virus. However, it has been shown, that dactinomycin inhibits the RNA synthesis of some poliovirus strains and mutants, especially at increased temperature (Kean et al., 1989; Racaniello & Meriam, 1986; Schaffer & Gordon, 1966; Tolskaya et al., 1968). Therefore, we compared the results described above with those obtained during pulse-labelling experiments in which the drug was added to virus-infected cells shortly before the addition of labelled nucleosides and subsequent extraction of RNA (data not shown). The two sets of results did not differ significantly, showing that reduced RNA synthesis could not be explained by the presence of dactinomycin throughout infection.

Discussion

This paper describes the first study of the effects of engineered point mutations introduced within the NTP-binding pattern of a picornavirus 2C protein. Using site-directed mutagenesis, four positions within the A motif and one position within the B motif of the putative NTP-binding pattern of the poliovirus 2C protein were individually mutated. Two or three different mutants were derived for each position. These were chosen such that each amino acid was replaced either by a very similar one or by less related ones. A special case was the Pro residue. Proline has no close counterpart, and it was substituted by three different, more or less similar, amino acids.

When Li & Baltimore (1988) introduced short in-frame insertions into the poliovirus 2C gene, viable mutants with mutations in the central conserved region of this protein were not obtained. In contrast, viable mutants with mutations in the C-terminal domain of the 2C polypeptide were selected. This probably reflects stricter structural or functional requirements within the middle region of the 2C protein than at its C terminus. Our data show that viable mutants can be obtained in which the middle region of the poliovirus 2C protein is affected, provided the mutations affect non-conserved amino acids. This is in agreement with reports of poliovirus mutants selected for resistance to guanidine, in which amino acid changes are located in the middle region of the 2C protein (Pincus & Wimmer, 1986; Pincus et al., 1986, 1987; Baltera & Tershak, 1989; K. M. Kean & H. Agut, unpublished data). The guanidine-sensitive phenotype of wild-type poliovirus remains basically unchanged for viable A motif mutants; virus production is totally abolished in the presence of 1 mM-guanidine hydrochloride (unpublished results).

The results reported here provide experimental confirmation of the importance of highly conserved amino acids in both the A and B motifs of the poliovirus 2C protein because all mutations at such positions are lethal for virus growth. In molecular genetic studies of other poliovirus proteins, such as the 3C protease, it has been shown that functionally important residues cannot be replaced, even by residues found in the equivalent positions of other picornaviruses (Kean et al., 1991). Pro and Thr are not conserved positions, neither in terms of nucleotide-binding proteins, nor among picornaviruses (see Fig. 1a). Replacement of the residue at these positions by one found in other picornaviruses resulted in a viable virus. This demonstrates that the region of the 2C protein studied is not subject to stringent conservation as a whole. Furthermore, the differential results concerning residues 131 and 133, and residues 135 and 136, respectively, suggests differential functional importance of different residues within a six amino acid stretch.

The lethal mutants proved to be defective in RNA synthesis, in agreement with existing data about the involvement of the poliovirus 2C protein in viral RNA synthesis. However, it should be noted that although in several cases RNA synthesis was not detectable at all by the method used, viable revertant viruses were obtained. It is conceivable that revertant viruses would emerge at low frequency, as in the case of Lys→Arg, Lys→Gly, Asp→Glu, Asp→Gly or Ser→Ala, as a result of errors introduced by T7 RNA polymerase in vitro. However, this could not be the case for the emergence of revertant viruses at high frequency, such as for Ser→Thr and especially Pro→Thr. The systematic appearance of revertants is highly suggestive of a residual low level of replication after transfection. We are currently trying to increase the sensitivity of the methods of detection of virus-specific RNA to address this question.

It is interesting to compare the effects of the mutations studied here with those observed in other NTP-binding

<table>
<thead>
<tr>
<th>Virus</th>
<th>Time post-infection (h)</th>
<th>Maximal RNA synthesis (%)</th>
<th>Level (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5-5</td>
<td>93</td>
<td>44</td>
</tr>
<tr>
<td>Thr110Ser</td>
<td>7</td>
<td>69</td>
<td>44</td>
</tr>
<tr>
<td>Thr131Ala</td>
<td>7</td>
<td>46</td>
<td>16</td>
</tr>
</tbody>
</table>

* Percentage of wild-type RNA synthesis at 37 °C.
proteins. Ser\textsuperscript{136}, being absolutely conserved among picornavirus 2C proteins, is replaced by Thr in the homologous protein of calcivirus as well as in the A motif of a number of other NTP-binding proteins (Gorbalenya & Koonin, 1989). Although the Ser\textsuperscript{136}→Thr mutation abolishes viral RNA replication, viable Ser\textsuperscript{136}→Thr mutants can be isolated, but only after an additional mutation (Asn\textsuperscript{140}→Tyr) has arisen.

In contrast, in the case of the Lys\textsuperscript{135}→Arg mutation, only primary site revertants to the wild-type amino acid were isolated. This is in agreement with data obtained for another NTP-binding protein, the \textit{wrbB} helicase of \textit{E. coli}, for which the replacement of the conserved Lys within the A motif by an Arg results in the functional inactivation of the protein (Seeley & Grossman, 1989).

Changes of Pro\textsuperscript{131} have different effects on virus viability and RNA synthesis depending on the amino acid replacement. The viable Pro\textsuperscript{131}→Ala mutant shows a decreased level of RNA synthesis, and the progeny exhibit a mixed-size plaque phenotype (Fig. 3). The non-viable Pro\textsuperscript{131}→Thr mutant gave rise to true revertants with an extremely high efficiency, whereas no revertants could be derived from another non-viable mutant, Pro\textsuperscript{131}→Asn. The failure to obtain viable revertants from Pro\textsuperscript{131}→Asn cannot be attributed solely to the fact that the restoration of the wild-type amino acid requires two nucleotide changes because such mutations occurred in two other cases (Lys\textsuperscript{135}→Ser and Asp\textsuperscript{177}→Gly), albeit at a very low frequency. It is possible that the Asn replacement is the only one described in this study which totally abolishes RNA replication. Pro\textsuperscript{131} could be regarded as a probable analogue of the functionally important Gly\textsuperscript{12} residue of the \textit{ras} oncogene protein (Levinson, 1986) because they both occupy the same position in the A motif. For the p21-ras proteins, a regulatory role has been determined for Gly\textsuperscript{12}. The majority of amino acid changes at this position were found to prevent GTP hydrolysis completely, rather than to interfere with GTP binding, apparently due to physical blockage of access to the catalytic site (Valencia \textit{et al}., 1991). Our data are consistent with the hypothesis that like Gly\textsuperscript{12}, Pro\textsuperscript{131} of the 2C protein could play a regulatory role. Effectively, the more drastic the amino acid change at this position, the more severe the effects on virus viability and the emergence of revertant viruses.

In conclusion, the data presented concerning the site-directed mutagenesis of five different positions of the putative nucleotide-binding pattern of the poliovirus 2C protein demonstrate the functional significance of the presence of this sequence. The results are fully consistent with its predicted functions of ATP binding and hydrolysis. Future experiments will be devoted to the demonstration of the enzymatic activities exhibited by wild-type 2C protein and by mutants such as those described here.

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