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Functional and Genetic Plasticities of the Poliovirus Genome: Quasi-Infectious RNAs Modified in the 5′-Untranslated Region Yield a Variety of Pseudorevertants

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Poliovirus RNA species with nucleotides 564 to 571 deleted or with a secondary structure domain (positions 564 to 629) replaced by a shorter irregular oligonucleotide have been engineered previously; these RNAs have been considered quasi-infectious (yielding a single late revertant plaque) and dead, respectively (E. Pilipenko, A. Gmyl, Y. Svitkin, S. Maslova, A. Sinyakov, and V. Agol, Cell 68:119–131, 1992). By using large amounts of these RNAs for transfections, revertant clones with a great variety of genetic changes (point mutations, insertions of foreign sequences, short or extended deletions) were isolated. The pattern of these changes supported the notion that an appropriately spaced oligopyrimidine-AUG tandem is important for efficient poliovirus RNA translation. Structural features within and around this tandem modulated the initiation efficiency. The functional and genetic plasticities of the poliovirus genome are briefly discussed.

The poliovirus RNA, a 7.4-kb molecule of mRNA sense polarity, shows a very high conservation upon growth under more or less constant conditions. The viral genomes in different populations of a given strain are remarkably similar. For example, genomic segments of several poliovirus strains have been sequenced by our group (17, 19, 21, 26), and very few, if any, differences from the sequences originally reported (11, 15, 20, 22) have been found, despite the numerous generations separating the virus lots studied. On the other hand, it is a common observation that poliovirus mutants with unfavorable mutations can, upon cultivation, readily correct the phenotype by accumulating genetic changes.

Thus, a variety of relatively fit clones have been selected from the mutants with insertions into the 5′-untranslated region (5UTR) of poliovirus RNA (18), which is known to harbor a control element promoting the cap-independent initiation of viral translation (for a review, see reference 1). The insertions were targeted to a locus in this element that is between two of its conserved motifs, an oligopyrimidine (box A) and a noninitiator AUG located at position 586; the box A-AUG distance was thus significantly increased. However, as a result of different deletions removing parts of the inserts or point mutations creating new AUGs, the revertant genomes restored a nearly wild type arrangement of these two motifs (18). That study demonstrated, among other things, (i) the possibility of fulfilling specialized function(s) of the translational *cis* element by diverse RNA structures and (ii) a great degree of intrinsic infidelity of poliovirus genome replication. The aim of the present work was to obtain new insights into both of these issues. (Preliminary results were presented at a meeting on translational control, Cold Spring Harbor, N.Y., 9 to 13 September 1992.)

First, we studied the capacity of the virus to restore its functions after a nearly unacceptable shortening (rather than lengthening) of the box A–AUG-586 spacing. To this end, we made use of a mutant plasmid, pPV1/Δ8, lacking poliovirus type 1 (PV1) RNA nucleotides (nt) 564 to 571 (18). The transcript of this plasmid (with the wild-type box A-AUG distance of 22 nt reduced to 14 nt) was a poor translational template in extracts from Krebs-2 cells; it was considered quasi-infectious since upon transfection of African green monkey kidney cells with the mutant RNA at 500 ng per flask (or 2 to 3 orders of magnitude higher than the standard amount), only a single, late-appearing plaque was observed (18). When transfections with high RNA doses (100 to 200 ng per flask) were repeated, solitary plaques appeared in 7 more flasks (out of 11 used in the experiments) after long incubation periods (10 to 17 days, compared with 2 to 3 days in the case of fully infectious RNA species). The material from each of these plaques was used for infection of fresh cultures; with poorly growing viruses, the passages were repeated. From heterogeneous-size plaques produced by each isolate, relatively large representatives were picked up, and an appropriate segment (positions ~450 to 720) of the genome of each of the isolates was sequenced (Table 1).

It is convenient to compare the character of reversions in the progeny of the deletion mutant studied here and in the descendants of the insertion mutants (18). In both cases, the engineered mutations altered the box A–AUG-586 spacing. The reversions tended to restore the wild-type arrangement of these motifs; this was achieved in one of three ways. The class I revertants acquired, by point mutations, a novel AUG at an appropriate distance from box A, either upstream or downstream of the resident AUG-586 in the progeny of the insertion or deletion mutants, respectively. In this study, a novel AUG was generated either just 3′ (in clone PV1/Δ8-82) from AUG-586 or after a 3-nt spacer (clones PV1/Δ8-91 and -107) (Table 1); thus, the novel AUGs lay at a more comfortable distance (17 or 20 nt) from box A. It is noteworthy

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TABLE 1. Properties of revertant viruses derived from PV1/Δ8 RNA

Plaque	Virus[a]	Sequence[b]	Box A-AUG distance (nt)	Plaque size[c]	Template activity[d]
		559 ↓ … 619 ↓ 717 ↓ … 747 ↓			
	PV1	UUUCCUUUUAUUUUAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUGUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	22	9	1.0
	PV1/Δ8	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUGUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	14		0.05[e]
GD801	112	UUUCC--------UAUUGUGGCUuCUUAUGGUGACAAUCACAGAUUaUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	14	2	0.28
GD803	91	UUUCC--------UAUUGUGGCUGCUUAUGGUGAugAUCACAGAUUGUUAUCAUAAAGCGg..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	20	4.5	0.16
GD808	107	UUUCC--------UAUUGUGGCUGCUUAUGGUGAugAUCACAGAUUGUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	20	4.5	0.21
GD807	82	UUUCC--------UAUUGUGaCUGCUUAUGaUGAuAAUCACAGAcUGUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	17	3.5	0.13
GD802	106	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGgUUaUUAUCAUAAGCGAA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	16	5.5	0.54
		↑ au			
GD804	77	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	16	5.5	0.44
		↑ au			
GD805	89	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	16	6	0.51
		↑ uu			
	88	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaUUAUCAUAAAGCGA..UAUUUCAAUCAGACAAUUGUAUCAUAAUGGG	23	8	0.9
		↑ auacgcuau			
	90	UUU--..-AUUUCAAUCAGACAAUUGUAUCAUAAUGGG	19[f]	4.5	0.21
GD806	74	UUUC---..------AAUCAGACAAUUGUAUCAUAAUGGG	19	5.5	0.4

[a] For the sake of brevity, the prefix PV1/Δ8 is omitted from designations of the revertant clones.

[b] Partial sequencing of RNA species isolated from the revertant viruses was done by a modification of the chain termination method, using avian myeloblastosis virus reverse transcriptase and appropriate synthetic oligodeoxyribonucleotide primers (17). The nucleotide differences from the wild-type (PV1) RNA are indicated in lowercase letters; deleted nucleotides are shown by dashes. The upstream and initiator AUGs are underlined by thin and thick lines, respectively. The following RNA segments were sequenced: positions 160 to 740 for PV1/Δ8-112; positions 140 to 720 for PV1/Δ8-91 and -77; positions 68 to 985 for PV1/Δ8-90 and -74; and positions ~450 to 720 for PV1/Δ8-107, -82, -106, -89, and -88. No mutations in other parts of the segments sequenced were observed.

[c] Average size in millimeters, measured on day 3.

[d] Measured in mRNA-dependent extracts of Krebs-2 cells as described in the legend to Fig. 1. Regions of the gel corresponding to the P1 band were excised, and radioactivity was counted in a liquid scintillation spectrometer. Template activities of the revertant RNA species were normalized to that exhibited by the wild-type PV1 RNA.

[e] Template activity of the RNA transcript rather than the viral RNA was measured; data are from reference 18.

[f] Distance between UUUCA and AUG.

that the AUGs in two independent clones were generated by transitions of two contiguous nucleotides, CA→UG.

In the class II revertants, the disturbed spacing between the two motifs was corrected either by deletions (in descendants of the insertion mutants) (18) or insertions (in progeny of pPV1/Δ8) (Table 1). Among revertants of the latter type, each of three independent clones (PV1/Δ8-106, -77, and -89) had a 2-nt insertion, and one clone (PV1/Δ8-88) acquired 9 foreign nt between box A and AUG-586. As a result, the box A-AUG distance was increased from 14 nt to 16 and 23 nt, respectively. In addition, many class I and class II revertants harbored diverse point mutations which may (see below) or may not be of physiological significance.

Finally, the class III revertants (PV1/Δ8-90 and -74 [the latter corresponded to GD-806 in reference 18]) had extended deletions (152 and 156 nt, respectively), which included the resident AUG-586, the entire conserved domain housing it, and most of the spacer between this domain and the initiator AUG-743. The box A-AUG tandem was restored by using the initiator codon in place of the lost cryptic AUG. Originally, viable poliovirus mutants similar to the class III revertants have been engineered in Nomoto's laboratory (7, 12); recently, the class III revertants were observed also by another group (5).

One revertant could not be assigned to any of these three classes: PV1/Δ8-112, a small-plaque former, had two point mutations in the region sequenced; their possible significance will be discussed below. This virus was stable enough to preserve its genotype upon at least three additional passages.

The reversions listed in Table 1 resulted also in a significant increase in protein-synthesizing activity (the original RNA transcript with the Δ8 deletion was translated at an efficiency of about 5% [18]), and there was a reasonably good correlation between the revertant's plaque sizes and RNA translatability (Table 1 and Fig. 1). The revertant RNA template activities varied from 13 to 90% compared with that of the wild-type RNA. This variation could be due in part to differences in the box A-AUG distance (the weakest [PV1/Δ8-82] and strongest [PV1/Δ8-88] templates have distances of 17 and 23 nt, respectively). This, however, was not the sole explanation; additional negative and positive effects seemed to be involved.

The revertants with newly acquired AUGs (PV1/Δ8-91, -107, and -82) all appeared to display anomalously low template activities, despite a quite appropriate box A-AUG distance (20 nt in two cases). A likely explanation for this anomaly lies in the fact that the novel AUGs emerged within a relatively good Kozak context (a purine in position −3 and an A in position +4). Such a motif, potentially able to promote initiation, could be expected to exert an adverse effect on the properties of the control element; indeed, acquisition of the initiator capacity and a decrease in plaque size of the appropriate mutant upon a similar improvement of the context of AUG-586 in wild-type poliovirus has recently been demonstrated by Wimmer's laboratory (28). On the other hand, mutation G-605→A could probably enhance the proficiency of the revertants with a relatively short box A-AUG distance (PV1/Δ8-77, -89, and -106). This mutation, present in the progeny of four independent plaques, including PV1/Δ8-112 (the only revertant without new AUG or insertions or deletions), is expected to destabilize the conserved structure of domain E; the other mutation found in PV1/Δ8-112 should exert a similar effect (Fig. 2). It was suggested previously that destabilization of the secondary structure around the AUG involved in the initial

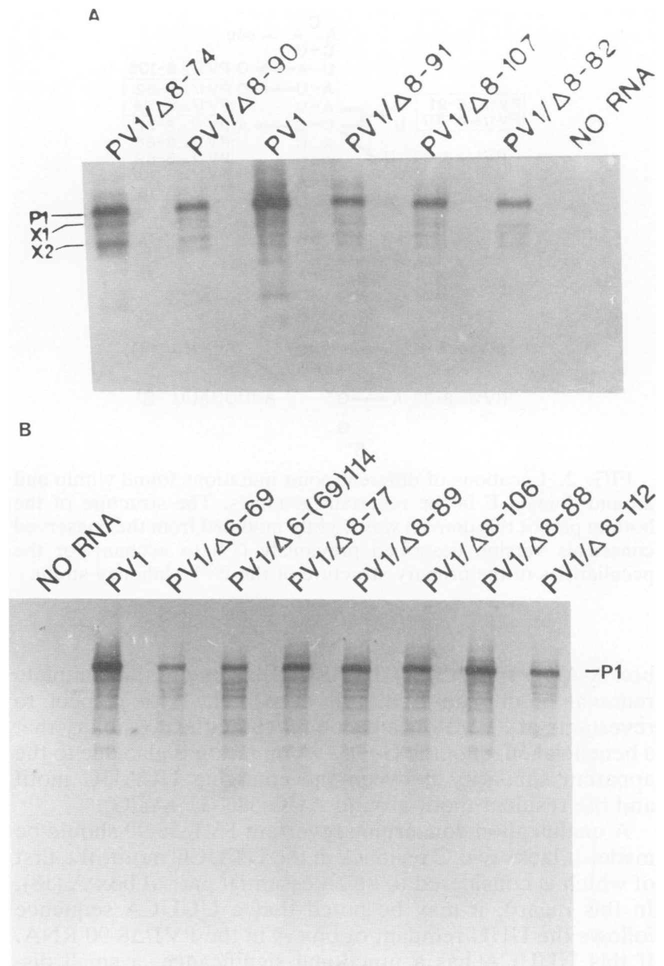

FIG. 1. Template activities of revertant RNAs. The RNA species isolated from the viruses were translated in extracts from Krebs-2 cells (30°C, 1 h, 20 μg of RNA per ml) essentially as described previously (18, 23). The labeled products were subjected to electrophoresis in sodium dodecyl sulfate-containing polyacrylamide slab gels and fluorography. (A) Revertant RNAs with AUG-generating point mutations (PV1/Δ8-91, -107, and -82) and extended deletions (PV1/Δ8-74 and -90). P1 designates the band corresponding to the precursor of capsid polypeptides; the nature of X1 and X2, directed by the RNA species with large deletions, is unknown. (B) Revertant RNAs with insertions (PV1/Δ8-77, -89, -106, and -88). Results obtained with the RNAs of the previously engineered mutant PV1/Δ6-69 (PV1/Δ6 in reference 18) and its revertant PV1/Δ6-(69)114 (see text) are also shown.

interaction with ribosomes could stimulate translation of certain mutant templates (18). The notion that the domain E destabilization stimulates translation of modified RNAs is further strengthened by the observation that a mutant lacking 6 nt in the box A–AUG-586 spacer, PV1/Δ6, is a small-plaque former (18); the only change found in the RNA of its revertant PV1/Δ6-(69)114 (exhibiting an increase in plaque size and RNA template activity; Fig. 1B) was transition U-603→C (not shown), which again is anticipated to destroy the contiguity of the stem in domain E (Fig. 2). It may also be speculated that a relatively high in vitro activity of an artificial template having the human rhinovirus 2 internal ribosome entry site with a significantly shortened

FIG. 2. Locations of different point mutations found within and around domain E in the revertant genomes. The structure of the bottom part of this domain was slightly modified from the conserved consensus version described previously (17) to account for the peculiarities of the primary structure of the PV1 Mahoney strain.

box A-AUG spacer (pXLJ 10-562 [3]) is due to the complete removal of domain E in this construct. With respect to revertants of PV1/Δ8, we cannot exclude the possibility that a beneficial effect of the G-605→A mutation is also due to the apparent similarity between the emerging UUAUU motif and the resident motif around AUG-586 (UUAUG).

A qualification concerning revertant PV1/Δ8-90 should be made. It lacks two C residues in the UUUCC motif, the first of which is considered to be an essential part of box A (18). In this regard, it may be noted that a UUUCA sequence follows the UUU remnant of box A in the PV1/Δ8-90 RNA. If this UUUCA has a functional significance, a small displacement of box A may perhaps be compatible with its rather strong position effect (16, 18). It also cannot be excluded that a decreased stringency in requirements for the box A position was somehow related to the absence, in PV1/Δ8-90, of AUG-586 and/or domain E with the adjoining spacer. Accumulation of additional polypeptide bands (X1 and X2) in the reactions directed by RNAs of PV1/Δ8-90 and -74 (Fig. 1) should be noted; the nature of these polypeptides is under study.

Encouraged by the recovery of a variety of revertants from the nearly dead PV1/Δ8 RNA, we reinvestigated the infectivity of a genome thought to harbor a lethal mutation. The transcript of plasmid pPV1/ΔE/24M had an AUG-lacking 24-nt-long insert in place of the entire domain E (Fig. 3); it served as a negative control in a previous study (18). This RNA should, however, be considered quasi-infectious since a subsequent high-RNA-dose transfection assay was successful and plaques were produced in several flasks; the plaques appeared relatively late (after 6 to 10 days) and were quite small (1 to 2 mm). The viral RNA from six plaques was polymerase chain reaction (PCR) amplified, and three PCR clones from each plaque were sequenced; in all cases, the RNA was found to contain an identical G-to-A transition downstream of the insert, generating an AUG 31 nt 3′ to box A (Fig. 3). Two more proficient independent clones were isolated from large plaques appearing upon further passaging of progeny of the small plaques. Both revertants (PV1/ΔE/24M-83 and -99) lost nucleotides from the insert, reducing the box A-AUG spacing to 27 and 23 nt, respectively; the latter genome acquired an additional point mutation of unknown physiological significance at position 517 (Fig. 3).

When larger segments of the 5UTRs of representatives of each class of revertants were sequenced, no additional mutations were found with the single exception of PV1/ΔE/24M-99 (see footnote *b* to Table 1 and the legend to Fig. 3). Furthermore, *Kpn*I-*Sca*I segments (positions 68 to 985) of cDNAs of revertants PV1/Δ8-74, -90, and -99 as well as *Bam*HI-*Bam*HI segments (positions 222 to 672) of PV1/Δ8-82 and -91 were generated and inserted into a full-length infectious cDNA of poliovirus; the clones recovered upon the transfection with the appropriate transcripts exhibited the expected phenotypes (unpublished observations). All of these data strongly suggested that the revertant phenotypes were due to the mutations noted in Table 1 and Fig. 3.

Thus, the RNA structures of descendants of pPV1/Δ8 and pPV1/ΔE/24M strongly support the importance of the properly arranged tandem of box A and an AUG and thus fit nicely the model of the control element's structure proposed earlier (18; see also references 8, 9, and 14). Furthermore, our results document an extraordinary plasticity of the translational control element, which retained its functional activity upon very different structural rearrangements.

It was interesting to monitor genetic changes in the course of evolution of individual revertant lineages. The possibility that mutations conferring the ability to form plaques found in the revertants had already been present in the RNA transcripts should be ruled out. Indeed, these transcripts produced plaques not only at a very low frequency but also after unusually long lags (plaques formed by the revertants themselves, though sometimes rather small, were always visible on day 3). The relevant segments (corresponding to positions 416 to 759 of the wild-type RNA) of the viral genomes in plaque GD805 and in subsequent populations of this clone were investigated by PCR amplification (Fig. 4). A nearly homogeneous band was observed in the materials from the original plaque (not shown) and from the virus formed during passage 1 (lane 2); the length of this material corresponded to the parental engineered genome and was 8 nt shorter than in the PV1 RNA (lane 4). The passage 2 population contained more heterogeneous RNA (lane 3); a band 2 to 3 nt longer than in the Δ8 parent predominated, but additional minor bands were also visible. The length of one of them was similar to or slightly greater than that in PV1, whereas the other, a doublet, was about 150 nt shorter.

The *Bam*HI-treated PCR-amplified segments were cloned and sequenced at each step of evolution (Table 2). Five PCR clones from plaque GD805 and three clones from the passage 1 material were found to be identical to each other, showing a single G-605→A transition. Among the 19 PCR clones obtained from passage 2, all retained this change and some had, in addition, an insertion of two (nine clones), three (two clones), or nine (two clones) bases. Since the pattern of PCR products suggested the presence of a significantly shortened version of the 5UTR in the material from passage 2 (Fig. 4, lane 3), which could lack the *Bam*HI site at position 672, the full PCR-amplified fragment (positions 416 to 759) was also cloned. Four clones were analyzed, and two forms of the class III revertants were detected; one was identical to that in PV1/Δ8-90 (Table 1), and the other had a 154-nt deletion 14 nt downstream of the engineered Δ8 deletion. Thus, a well-spaced tandem of box A and the initiator AUG was again created (Table 2). The data presented in Table 2 demonstrated that during the evolution of the progeny of plaque GD805, the mutation destabilizing domain E appeared first, and the insertions (correcting the box A-AUG

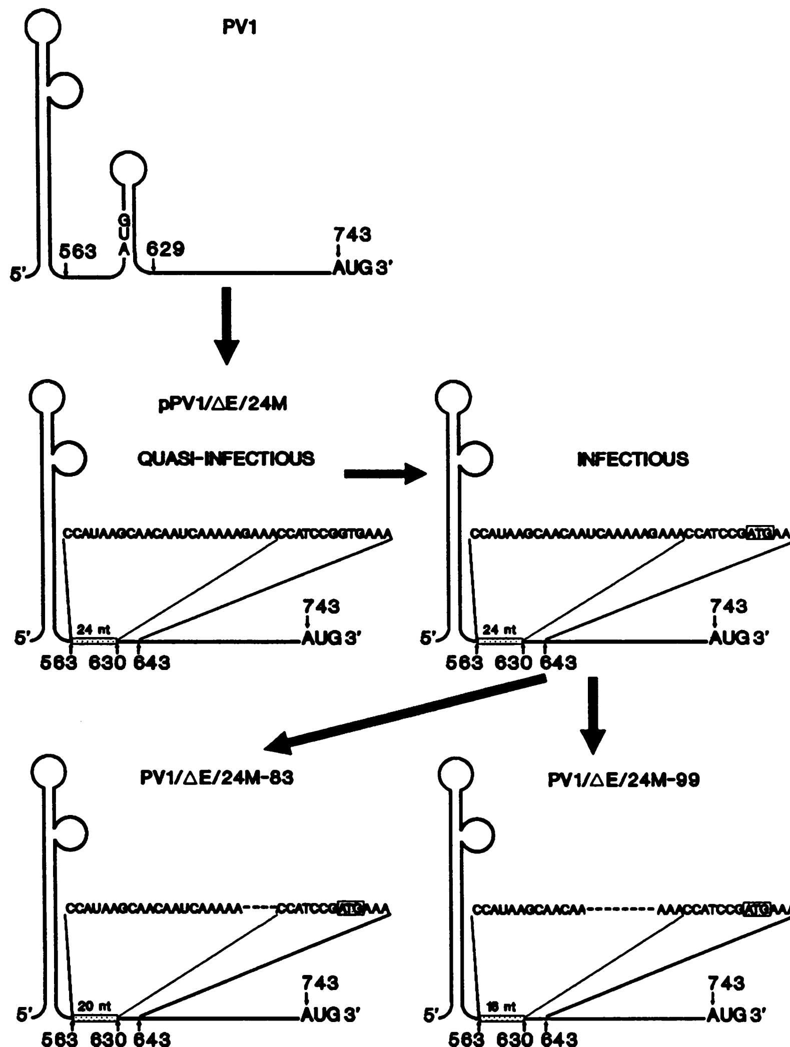

FIG. 3. Evolution of the progeny of quasi-infectious PV1/ΔE/24M RNA. RNA segments between positions 450 and 720 and positions 68 and 985 were sequenced in PV1/ΔE/24M-83 and -99, respectively. In addition to the mutations indicated, the transition C-517→U was found in the latter case. See text for other details.

distance) or large deletions (creating a novel box A-AUG tandem) were acquired subsequently.

A similar analysis was performed with the material from another primary plaque (GD806) generated by the pPV1/Δ8 RNA, and all of the five clones studied revealed the presence of a large deletion identical to that found in PV1/Δ8-74 (Table 1) (data not shown). Therefore, the large deletion was, in this case, a primary genetic change.

Thus, the adaptation of the viral genome may result from diverse replicative errors. The poliovirus RNA-dependent RNA polymerase, being devoid of proofreading activity, is known to produce point mutations at a relatively high rate (references 4, 6, and 27 and references therein). Quite unexpectedly, comparable frequencies of deletions and insertions on the one hand and point mutations on the other were observed in proficient revertants during this study and previous (18) studies. Since the only changes that accumulated were those that resulted in an improvement of the impaired function of the translational *cis* element, there are reasons to believe that similar alterations occurred over the entire genome but, having no selective advantage, were not fixed. Thus, the frequency of deletions and insertions during viral growth is probably very high.

Deletions and insertions are likely to arise as a result of jumping of the viral RNA polymerase together with the nascent strand to another place on the same or another RNA template. If the jump involves homologous parting and anchoring sites on the two templates, homologous recombination could occur (10, 21, 26); if, however, the anchoring site is selected erroneously, both deletions and insertions should be generated (13, 21). The present results suggest that the replication of poliovirus RNA proceeds in a less processive mode and that jumping is less correct than might be expected.

The rules, if any, controlling the choice of the both parting and anchoring sites during such jumps remain to be elucidated. In some cases, short (2- to 4-nt long) repeats flanking the deleted segment could be detected, but exceptions to this rule exist. Moreover, if any individual deletion could in principle be due to a single incorrect jump, some of the insertions observed cannot be explained in such a simple way. The insertion of two U's in the PV1/Δ8-89 RNA could

TABLE 2. Genomes detected in plaque GD805 and its descendants[a]

Passage no. or virus	No. of clones: Investigated	No. of clones: With a given sequence	Sequence	Box A-AUG distance (nt)
0	5[b]	5	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG	14
1	3[b]	3	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG	14
2	19[b]	6	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG	14
		2	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG ↑ guu	17
		9	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG ↑ uu	16
		2	UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUaU..UAUUUCAAUCAGACAAUUGUAUCAUAUG ↑ auacgcuau	23
2	4[c]	3	UUU--..-AUUUCAAUCAGACAAUUGUAUCAUAAUG	19[d]
		1	UUUCC--------UAUUGUGGCUGCUU---------------------..-------------------UAUCAUAAUG	21
			559 ↓ ... 606 ↓ 717 ↓ ... 745 ↓	
PV1			UUUCCUUUUAUUUUAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUGU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG	22
PV1/Δ8			UUUCC--------UAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUGU..UAUUUCAAUCAGACAAUUGUAUCAUAAUG	14

[a] PCR amplification of the material from plaque GD805 and its descendants was performed as described in the legend to Fig. 4. After cloning (see footnotes *b* and *c*), the DNA was sequenced by the chain termination method, using the Klenow fragment of *E. coli* DNA polymerase I. Sequences of the appropriate segments of PV1 and PV1/Δ8 are given at the bottom for comparison.

[b] The *Bam*HI-treated PCR-amplified segment (corresponding to positions 416 to 672) was cloned into pBS M13(−) (Vector Cloning Systems, San Diego, Calif.) between the *Ecl*136II (a *Sac*I isoschisomer producing blunt ends; Fermentas, Vilnius, Lithuania) and *Bam*HI sites.

[c] The full PCR-amplified segment (positions 416 to 759) was cloned into the *Ecl*136II site of the same vector.

[d] See Table 1, footnote *f*.

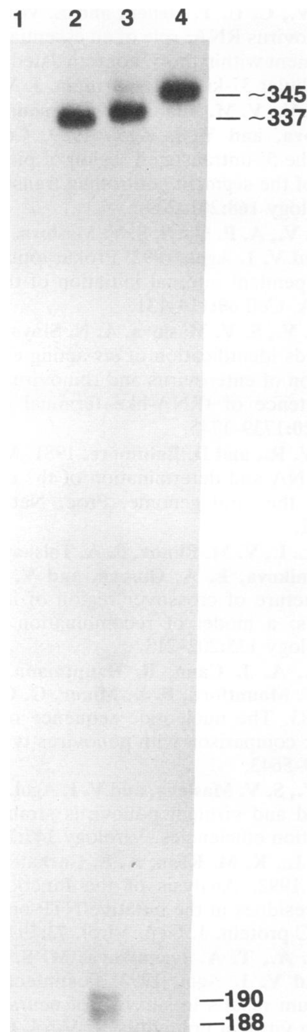

FIG. 4. Length polymorphism of PCR-amplified fragments of the progeny of the GD805 plaque induced by the pPV1/Δ8 transcript. Plaque GD805 was designated passage 0. The supernatant fluid from the infected cells at passages 1 and 2 (200 μl) was subjected to phenol deproteinization in the presence of sodium dodecyl sulfate. The RNA was reverse transcribed at 40.5°C for 2 h in 30 μl of a mix containing 50 mM KCl, 8 mM $MgCl_2$, 10 mM EDTA, 50 mM Tris-HCl (pH 8.3), 0.2 mM each of the four deoxynucleoside triphosphates, 5 U of the avian myeloblastosis virus enzyme, and 10 ng of the oligodeoxynucleotide primer complementary to positions 744 to 759 of the viral RNA. The virus-specific DNA (3 μl of the reaction mixture described above) was subjected to 25 cycles of PCR amplification (95, 50, and 72°C; 30 s for each step) in a DNA Thermal Cycler (Perkin-Elmer Cetus), using 2.5 U of *Taq*I polymerase and other reagents from the Perkin-Elmer Cetus DNA amplification reagent kit; the primers (1 μg per sample) corresponded to positions 744 to 759 (antisense) and 416 to 435 (sense) of the viral RNA. Electrophoresis was carried out in a sequencing gel; the lengths (in nucleotides) were calculated from a ladder run on the same gel. Lanes: 1, control (cultural fluid from uninfected cells); 2 and 3, material from passages 1 and 2, respectively; 4, material from PV1-infected cells.

be due to the polymerase slippage at an existing A residue in the negative-strand template, but the appearance of an AU at the same place in the PV1/Δ8-106 and -77 RNAs could not be explained in this way. Moreover, the 9-nt insert in the pPV1/Δ8-88 genome has no counterparts in the vicinity of the locus of insertion (although there is such a sequence at positions 1139 to 1147 of the viral RNA, the insert could also have derived from a cellular RNA). Multiple acts of polymerase jumping should be invoked to explain such rearrangements.

Taking into account the error rate (4, 6, 27), the viral replication machinery should produce many thousands of RNA copies before a favorable mistake at a specific position can appear. Hence, some viral RNA polymerase should be synthesized by the original mutant genome prior to this mutational event. We infer that quasi-infectious RNA species can serve as translational templates, though inefficiently. Thus, upon entering a susceptible cell, such RNA molecules are likely to trigger a very slow infectious process lasting for many days or even weeks. The infected cell is not necessarily lysed, because the concentration of yet undefined virus-specific components may not reach a critical threshold (25). When the replication defect is corrected by a mutation, viral reproduction is activated and plaque-forming progeny are generated. The probability of acquiring further and diverse favorable mutations is increased tremendously. Similar scenarios could be applicable not only to mutants with defects in translation initiation but also to any other quasi-infectious mutants (24).

In light of these results, it seems appropriate to distinguish more strictly lethal and quasi-infectious mutants. One should take into account that higher amounts of quasi-infectious RNAs are required to obtain plaques and that the plaques themselves tend to appear after prolonged incubation and at irregular time intervals. Since these points are not always considered, some mutant genomes classified in the literature as having lethal defect may actually be quasi-infectious (as was the case for pPV1/ΔE/24M).

Taken together, the genetic stability of poliovirus mentioned in the introductory remarks and infidelity of viral replication suggest the existence of a remarkable selective power of the host cells in culture (2).

The burst of genetic variants observed in the progeny of mutants with significant functional defects may mimic branchpoints in the evolution of viruses, whereupon significantly different viral species emerge. Obviously, the poor compatibility between the host and the infecting virus would favor the emergence of new variants, because a more competitive partner would not outgrow the newly emerging weak variants. Eventually, the evolving variants could be stabilized by further mutations. The stepwise emergence of relatively fit and stable descendants of PV1/ΔE/24M may be regarded as a model for such evolution.

The direction of the evolution will depend not only on the character of the mutation but also on the properties of the hosts available. Upon selection in tissue culture, large deletions in the 5UTR readily accumulated, even though such deletions have never been encountered in any natural enterovirus. To explain this apparent contradiction, we suggested that these deletions resulted in a loss (or severe impairment) of the mutant's ability to grow in the natural host, e.g., neural cells, while the reproductive capacity in the standard cell lines remains essentially unaltered (2a). Preliminary data obtained in this laboratory are in full accordance with this suggestion.

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