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PCR Engineering of Viral Quasispecies: a New Method To Preserve and Manipulate Genetic Diversity of RNA Virus Populations

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A PCR-based method for the controlled manipulation of individual genomic sites of poliovirus with concomitant preservation of the sequence heterogeneity of the rest of the genome is proposed. The new approach can be used for the creation of stable DNA repositories of populations of extremely heterogenous RNA viruses and may have implications for live vaccine technology.

Demonstration of the infectivity of poliovirus cDNA by Racaniello and Baltimore (21) was a groundbreaking discovery in poliovirus genetics. It provided a powerful tool for studying the biology of poliovirus and opened up the prospect of using poliovirus as a vector for the expression of foreign sequences (2), thereby creating a new field of RNA biotechnology. However, the use of cDNA clones for engineering RNA viruses has an inherent limitation, because it discounts the noncoherent nature of RNA virus populations. RNA viruses possess extreme sequence heterogeneity and are, therefore, described as "quasispecies" (10, 16). The sequence diversity of RNA viruses is critical for understanding not only their evolution but also of such properties as pathogenicity (14).

Regardless of how small the magnitude of this sequence diversity may be, it cannot be ignored if the relationship between genomic sequence and viral properties is to be studied. For instance, we found that all the batches of oral poliovirus vaccine (OPV) of all three serotypes contain small but measurable amounts of neurovirulent mutations (8). In type 3 OPV, an increase of the C-472 mutant content from 0.5 to 0.8%, the level typical for regular vaccine lots, to 1.2 to 1.5% results in an unacceptably high neurovirulence in monkeys (5, 8). Propagation of the Sabin vaccine strains of poliovirus under different conditions in vitro and in vivo created distinct mutation profiles of the virus (22, 23, 26). Thus, the properties of a particular virus stock depend not only on the prevailing consensus sequence of its genomic RNA but also on the subtle differences in the mutational composition of the viral quasispecies which reflect its passage history. Clones derived from individual RNA molecules are likely to lose some genetic information contained in a quasispecies in the form of sequence diversity and often contain random mutations.

The preservation and manipulation of the sequence diversity of RNA viruses are consequently important objectives. Recent improvements in PCR technology allow for a significant increase in the size of amplified DNA and create the possibility of making substantial amounts of full-length poliovirus cDNA without cloning. In this communication, I report that the virus recovered from poliovirus cDNA amplified by extra-long PCR (XL PCR) retains the sequence heterogeneity of the source virus used to make the cDNA. DNA copies of heterogenous virus samples can be prepared and stably maintained in vitro for subsequent rederivation of live virus representing a swarm of microvariants present in the original sample. Point mutation constructs (including partial nucleotide substitutions) and recombinants that retain sequence diversity and mutational profiles of the parental virus(es) were obtained by PCR-based site-directed mutagenesis.

cDNA was synthesized on an RNA template isolated from a CsCl-purified poliovirus by using SuperScript II reverse transcriptase and the primers T_{29} C CTCCGAATTA AAGAAA AATT for type 1 poliovirus (A1 primer), T_{30} CCCCGAATTA AAGAAAAATT TACCCCTACA for type 2 (A2 primer), and T_{30} CCTCCGAATT AAAGAAAAAT TTACCCCTAC for type 3 (A3 primer). XL PCR amplification was performed with the Perkin-Elmer XL-PCR kit and sense primer S1 (GCGGC CGCTA ATACGACTCA CTATAGGTTA AAACAGCTCT GGGGTTG), containing the T7 RNA polymerase promoter (underlined), or primer S2 (ACCGGACGAT TTAGGTG ACA CTATAGTTAA AACAGCTCTG GGGTTG), containing the SP6 RNA polymerase promoter (underlined), and antisense primer A1, A2, or A3. The hot-start PCR procedure consisted of incubation for 30 s at 94°C followed by 30 cycles each consisting of 15 s at 94°C and 10 min at 65°C and then by incubation for 20 min at 72°C. Figure 1 shows the products of the individual steps in preparing full-length DNA and the transcription of PCR products with T7 RNA polymerase (MEGAscript kit; Ambion) into the RNA that was then transfected into HEp-2 cells (25) to rederive infectious virus. Quantitation of mutants was performed by mutant analysis by PCR and restriction enzyme cleavage (MAPREC) (6, 8, 22, 26). Briefly, a DNA segment was made by PCR amplification of viral cDNA with partially mismatched primers, creating restriction sites that were affected by the mutation under study. The use of a partially mismatched primer(s) allows quantitation of practically any mutation, even those that do not affect naturally occurring restriction sites. Mutant quantitation was performed by determining the amount of DNA susceptible to restriction enzyme digestion after separation of radiolabelled fragments in a polyacrylamide gel and analyzing radioactivity with a beta-imager. The method is very sensitive and allows accurate quantitation of mutations present at levels below 1%. The tables present averages of at least two parallel determinations.

To see whether the virus recovered from XL PCR-amplified DNA contained the same level of sequence heterogeneity as the source virus, I compared the contents of mutants at different steps of the procedure. In Sabin polioviruses of types 2 and

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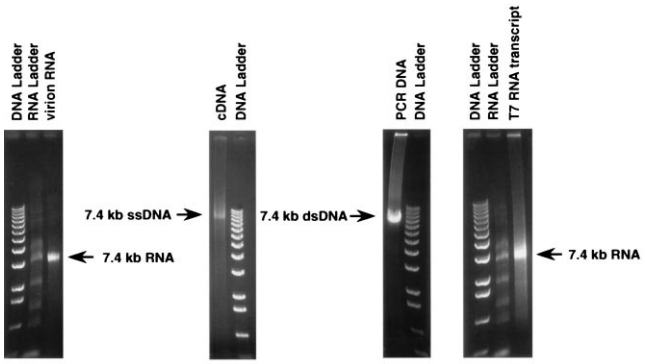

FIG. 1. Agarose gel analysis of poliovirus RNA and DNA products synthesized in different steps of the full-length PCR engineering protocol. Shown are (left to right) source virion RNA, full-length cDNA, XL PCR-amplified full-length double-stranded DNA (dsDNA), and in vitro T7 RNA transcript. Separation was performed with a precast Reliant gel system (1% Seakem Gold agarose; FMC BioProducts) and TAE buffer. After electrophoresis, the gels were stained with ethidium bromide. A 1-kb DNA ladder and an RNA ladder (BRL/Life Technologies) were used as molecular weight standards. ssDNA, single-stranded DNA.

3, the respective neurovirulent mutations A-481→G and U-472→C were tested. Table 1 shows that the content of these mutations remained unchanged after a full cycle of RNA→DNA→RNA conversions and infectious virus rederivation. DNA amplified on a template of homogenous cDNA clones was used as a control; some low apparent content of revertants in these samples is probably due to nucleotide misincorporation by *Taq* DNA polymerase during mutant quantitation by mutant analysis by PCR and restriction enzyme cleavage (18).

Two samples of the type 1 poliovirus with neurovirulent mutations at two complementary nucleotides (G-480→A and U-525→C) were tested. One sample contained a small amount of the revertants, while the other had a high content (higher passage level). The first two lines in Table 2 illustrate the stability of these mutations throughout the rederivation procedure.

A variety of PCR-based methods have been proposed for site-directed mutagenesis and in vitro recombination (15, 19). Point mutations at nucleotides 7427 and 7441 in the 3′ untranslated region of the Sabin 1 poliovirus were recently shown to mutate both in vitro and in vivo (22), but their role remains unknown. XL PCR amplification of type 1 poliovirus cDNA (made on a template of the low-mutation virus discussed above) with mutagenic primers containing C-7427 and A-7441 allowed the generation of virus strains which had complete substitutions at these sites but which retained the original sequence heterogeneities at nucleotides 480 and 525 (Table 2). Therefore, the ability to manipulate individual genomic sites without affecting sequence heterogeneity in the rest of the viral RNA opens the way for the study of the phenotypic expression of mutations within a specific mutational context of a particular virus strain.

TABLE 2. Content of A-480 and C-525 mutants in original and rederived samples of the Sabin 1 poliovirus[a]

Sample	Mutation	Content (mean % ± SD) of mutants in:	
		Source virus	Rederived virus
OPV 1 low[b]	A-480	0.25 ± 0.04	0.29 ± 0.03
	C-525	0.71 ± 0.62	0.63 ± 0.06
OPV 1 high[b]	A-480	2.35 ± 0.13	1.94 ± 0.11
	C-525	2.11 ± 0.04	1.75 ± 0.04
C-7427 mutant	A-480	0.25 ± 0.04	0.35 ± 0.01
	C-525	0.71 ± 0.62	0.51 ± 0.01
C-7441 mutant	A-480	0.25 ± 0.04	0.29 ± 0.03
	C-525	0.71 ± 0.62	0.50 ± 0.03

[a] Results of at least two parallel determinations are presented.
[b] Two virus samples at different passage levels (low and high) were used for the procedure.

Next, I attempted to create virus stocks with partial substitutions at specific genomic sites by using PCR mutagenesis of selected genomic fragments followed by PCR recombination to restore full-length viral DNA. I prepared mixtures of an antisense primer complementary to nucleotides 460 to 490 of type 2 poliovirus having vaccine-specific A-481 and a similar primer containing the neurovirulent G-481 mutation. These mixtures were used together with the S1 primer to amplify a segment of poliovirus DNA spanning nucleotides 1 to 490. Then, these T7 promoter-containing and predominantly single-stranded DNA segments (made by asymmetric amplification) were recombined in vitro with full-length type 2 poliovirus DNA having the SP6 promoter (made by XL PCR with S2 and A2 primers). This PCR recombination was carried out by coamplification of these DNAs with the S1 and A2 primers. A full-length PCR product was transcribed with T7 RNA polymerase, and the resulting RNA was transfected to rederive live viruses containing various amounts of G-481 (Table 3). Therefore, the proposed method of quasispecies engineering allows the creation of viral stocks with partial substitutions at specified genomic sites and enables the study of quantitative effects of different mutations on the phenotypic properties of the virus.

Unlike more traditional methods of genetic engineering based on the cloning of viral cDNA in recombinant plasmids, the new PCR technique described in this communication has several important advantages that I believe make it the method of choice for RNA viruses. Because RNA viruses are quasispecies, their properties cannot be completely described by the consensus sequence of their genomes alone; the full charac-

TABLE 1. Content of revertants in samples from intermediate steps of the Sabin 2 and Sabin 3 poliovirus rederivation procedure[a]

Sample	Mutation tested	Content (mean % ± SD) of mutants in:			
		Source material[b]	XL PCR DNA	T7 transcript	Rederived virus
Sabin 2 plasmid	G-481	0.06 ± 0.01	0.10 ± 0.03	0.07 ± 0.01	0.14 ± 0.02
Sabin 2 virus	G-481	1.18 ± 0.04	1.30 ± 0.08	1.31 ± 0.17	1.22 ± 0.12
Sabin 3 plasmid	C-472	0.15 ± 0.03	0.11 ± 0.01	0.13 ± 0.01	0.21 ± 0.04
Sabin 3 virus	C-472	0.81 ± 0.13	0.93 ± 0.03	1.04 ± 0.11	0.94 ± 0.03

[a] Results of at least two parallel determinations are presented.
[b] Poliovirus cDNA-containing plasmid or viral cDNA.

TABLE 3. Generation of type 2 point mutation constructs with various contents of the G-481 mutation

% G-481 primer in a G-481–A-481 mixture	Content (%) of G-481 mutation in[a]:			
	Short PCR DNA[b]	XL PCR DNA	T7 transcript	Rederived virus
0.5	0.50	0.62	0.41	0.66
2	2.34	2.49	2.76	2.47
5	4.80	4.74	4.65	5.86
10	10.85	9.80	7.66	9.47
90	87.74	79.02	80.12	81.68

[a] The source virus contained 1.18% G-481.
[b] Segment of DNA spanning nucleotides 1 to 490 in the 5′ untranslated region of the type 2 poliovirus.

terization of an RNA virus requires a knowledge of its fine genetic composition (quantitative mutation profile). This mutation profile depends on the distance from the clonal event, conditions of cultivation that favor accumulation of certain mutations, and chance selection by bottlenecking.

The presence of a variety of neutral and slightly deleterious mutations in a viral population not only constitutes a mutational burden that reduces the overall replicative capacity of the virus but also increases the effective informational capacity of RNA genomes, allowing the virus to adapt to changing growth conditions by providing preexisting genetic variants. It has been shown that the reduction of viral population complexity by serial bottlenecking or clonal events often results in a loss of fitness due to the so-called "Muller ratchet" mechanism (4, 9, 11, 12) while the fitness of viral variants increases during replication in a diverse population (13, 20). This observation means that competition within diverse populations is essential for maintaining and increasing the fitness of a virus and provides a driving force for viral evolution. Therefore, the genetic stability of a homogeneous cloned virus can be different from the genetic stability of a naturally diverse viral population with the same consensus sequence.

Despite the proven usefulness of cloning to reduce viral variability for simplified structural and functional analysis of RNA genomes, the ability to preserve and manipulate a whole variety of viral microvariants opens up a new dimension in the studies of RNA viruses. It allows mapping and identification of new genetic loci even for mutations present at such low levels that they escape identification by conventional techniques that involve cloning.

This new approach might also be used to create a stable repository of seed viruses for the production of live vaccines. While some live vaccines are produced from plaque-purified stocks (24), others were originated without cloning and may be extremely heterogeneous (1). But even plaque-purified vaccines like OPV are not homogeneous and contain measurable amounts of various mutations (7, 8, 22, 23, 26). It was recently proposed that recombinant plasmids carrying complete copies of poliovirus cDNA be used as stable repositories of seed virus (17). Since the properties and genetic stability of homogeneous vaccine stocks may differ from those of heterogeneous stocks, use of the proposed PCR method to create DNA repositories of validated seed viruses would alleviate concerns about the loss of important microheterogeneity in live viral vaccines.

PCR is a relatively error-prone method for DNA amplification and can produce incidental mutations when PCR products are subjected to cloning. The intrinsic error rates of the most commonly used *Taq* DNA polymerase is 10^{-4} to 10^{-5} (27). DNA polymerases used for XL PCR amplification possess much higher fidelity, with misincorporation rates of only 10^{-6} to 10^{-7} (3), which far exceeds the fidelity of poliovirus RNA replication (error rates of 5×10^{-3} to 7×10^{-4}) (28, 29). In addition, since no cloning is involved in the proposed method, the relatively rare mutations that occur during reverse transcriptase PCR amplification are likely to be randomly distributed and, therefore, have a limited contribution to the mutational background. Unlike mutations accumulating during virus replication in cells, mutations occurring during the reverse transcriptase PCR procedure are not subjected to natural selection, and therefore lethal and deleterious mutations will have more chances to persist in a population. However, since RNA transfection and virus growth are integral parts of the suggested method, the entire procedure of XL PCR rederivation of the virus is likely to be similar (in its effect on mutational composition) to a cell culture passage of the virus. Therefore, rederived virus is not an exact replica of the original virus batch but rather the closest possible approximation, similar to the next passage of the batch. Just as virus stocks produced under the well-controlled conditions of vaccine manufacturing have slightly different mutational compositions, the mutational profiles of the virus batches rederived by the XL PCR procedure can also be somewhat variable. In my experiments, however, I did not observe significant changes in the contents of mutations after a complete PCR rederivation cycle.

In conclusion, a method that preserves genomic heterogeneity and permits the manipulation of diverse populations of RNA genomes in vitro in many cases would be a more adequate tool for the study of the genetics of RNA viruses. Just as cDNA cloning of RNA viruses enabled molecular genetic studies of discrete viral strains, the proposed method would allow genetic manipulations of individual viral stocks.

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