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The Mechanism of RNA Recombination in Poliovirus

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Summary

We have investigated RNA recombination among poliovirus genomes by analyzing both intratypic and intertypic recombinant crosses involving the same defined genetic markers. Sequence analysis of the recombinant junctions of 13 nonsibling intertypic recombinants showed that intertypic RNA recombination is not site-specific, nor does it require extensive homology between the recombining parents at the crossover site. To discriminate between breaking–rejoining and copy choice mechanisms of RNA recombination, we have inhibited the replication of the recombining parents independently and found opposite effects on the frequency of genetic recombination in intratypic crosses. The results strongly support a copy choice mechanism for RNA recombination, in which the viral RNA polymerase switches templates during negative strand synthesis.

Introduction

Poliovirus is a positive strand virus whose genome consists of a single RNA molecule, approximately 7500 nucleotides in length, including a poly(A) stretch of variable length at the 3' terminus. Replication and transcription of poliovirus RNA are accomplished by a virally encoded RNA-dependent RNA polymerase (Baltimore et al., 1963). The genomic RNA molecules serve as templates for synthesis of full-length negative strands of RNA; these in turn encode the positive strand RNA molecules of the progeny virus. Cells coinfected with two different temperature sensitive mutants of poliovirus or other picornaviruses release wild-type virus at a low frequency (Hirst, 1962; Ledinko, 1963; Pringle, 1965), raising the possibility that recombination can occur among RNA genomes, with no involvement of a DNA intermediate. By contrast, RNA bacteriophage have not been observed to undergo genetic recombination (Horiuchi, 1975).

The existence of RNA recombination in picornaviruses has been convincingly demonstrated by further genetic analysis (Cooper, 1968, 1977; Lake et al., 1975; McCahon

et al., 1977) and by biochemical studies, showing that putatively recombinant viruses display inheritance from both parents of viral proteins (Romanova et al., 1980) and of viral RNA (King et al., 1982). RNA recombination has been used as a tool to generate genetic maps of picornaviruses (Cooper, 1977; Tolskaya and Kolesnikova, 1977; McCahon et al., 1977), and to localize heritable traits such as resistance to guanidine (Emini et al., 1984; Saunders et al., 1985) and neurovirulence (Agol et al., 1985). However, few experiments have directly addressed the mechanism by which genetic exchange between poliovirus RNA molecules occurs.

Two general classes of mechanism can be invoked to explain the phenomenon of RNA recombination. The first, molecular exchange, is similar in concept to the pairing, breaking, and rejoining events in homologous recombination of DNA. The enzymatic and autocatalytic splicing reactions that generate many eukaryotic messenger RNAs exemplify breaking and joining mechanisms causing RNA rearrangements. The second mechanism, a "copy choice" proposal, suggests that the viral RNA-dependent RNA polymerase, having initiated replication on one viral RNA, could switch templates and continue replication on a second viral RNA molecule. The concept that genetic recombination in DNA generally occurs by breaking and rejoining of parental DNA molecules is well accepted currently. It is of interest, however, that until 30 years ago most geneticists believed that DNA recombination proceeded by a copy choice mechanism (Belling, 1933; Hershey and Rotman, 1949). This idea was dispelled by the discovery that phage lambda recombinants contain atoms physically derived from both parents (Meselson and Weigle, 1961).

We have attempted to understand the mechanism of RNA recombination in poliovirus by studying recombinant crosses between defined genetic markers. The availability of an infectious cDNA replica of the poliovirus genome (Racaniello and Baltimore, 1981a) facilitates the construction and characterization of defined mutants (Bernstein et al., 1985; Sarnow et al., 1986), and the complete nucleotide sequence of poliovirus RNA (Kitamura et al., 1981; Racaniello and Baltimore, 1981b) makes it possible to define the sequence changes that correlate with the inheritance of more classical genetic markers such as guanidine resistance (Pincus et al., 1986; this work).

Using characterized genetic markers, we have obtained RNA recombinants resulting from genetic crosses between polioviruses of different serotypes. Sequence analysis of 13 nonsibling recombinants revealed the apparently random nature of the crossover points. In addition, to distinguish between breaking–rejoining and copy choice models of RNA recombination, we have tested the effect of inhibiting the replication of the parental viruses independently in intratypic genetic crosses. These experiments have direct implications for the mechanism of RNA recombination.

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Table 1. Characteristics of Genetic Markers

Virus	Location of Mutation	Coding Change	Description	Ratio of 32°C Titers with/without Guanidine	Ratio of Titers 39°C/32°C
Wild type			Mahoney serotype 1	3×10^{-6}	2
3NC-202	Nucleotide #7387	Insertion of GGTTAACC	Temperature sensitive mutation in 3'noncoding region	5×10^{-6}	2×10^{-3}
3NC-202gua^D	ND	ND	Selected from 3NC-202 at 32°C in 0.5 mM guanidine	2×10^2	ND
3NC-202gua^R	Nucleotide #7387 Nucleotide #4653 (2C codon #187)	Insertion of GGTTAACC AUG(met) to UUG(leu)	Selected from 3NC-202 gua^D at 32°C without guanidine	1	4×10^{-7}
	Nucleotide #4853 (2C codon #250)	GUC(val) to GCC(ala)			
R111	Nucleotide #4653 (2C codon #187)	AUG(met) to UUG(leu)	Intratypic recombinant between 3NC-202gua^R and wild-type Mahoney	2×10^2	1[a]

Temperature-sensitive poliovirus mutant 3NC-202 was constructed by linker insertion mutagenesis and has been characterized by Sarnow et al. (1986). Nucleotide sequences of the 2C regions of the 3NC-202gua^R virus and the intratypic recombinant R111 virus were determined, as described in Experimental Procedures, and compared with the known sequence of Mahoney type 1 poliovirus (Racaniello and Baltimore, 1981b). The ratios of plaque forming units of each viral isolate in the presence and absence of 0.5 mM guanidine, and at 39°C and 32°C, were determined by plaque assay.
[a] The ratio of titers at 39°C and 32°C of R111 was determined in the presence of guanidine.
ND, not determined.

Results and Discussion

Genetic Markers Used in Recombinant Crosses

3NC-202 (previously designated PTH7387) is a temperature sensitive poliovirus mutant that is defective in RNA replication (Sarnow et al., 1986) and displays a plaque reduction of 1000-fold at the nonpermissive temperature (Table 1). The cDNA encoding 3NC-202 virus contains an 8 bp insertion in the sequences corresponding to the 3' noncoding region of the viral genome (Sarnow et al., 1986; Table 1); the associated temperature sensitive defect in the initiation of negative strand synthesis (P. Sarnow, K. Kirkegaard, and D. Baltimore, unpublished data) cannot be complemented by viruses bearing mutations in various viral proteins (H. Bernstein, personal communication).

Guanidine resistant poliovirus mutants have been mapped by genetic and biochemical means to protein 2C, near the middle of the poliovirus genome (Saunders and King, 1982; Pincus et al., 1986; for nomenclature of picornavirus proteins, Rueckert and Wimmer, 1984). Guanidine hydrochloride at very low concentrations specifically inhibits the replication of wild-type poliovirus (Caliguiri and Tamm, 1968; Baltimore, 1968). When poliovirus is grown in the presence of 0.5 mM to 2 mM guanidine hydrochloride, however, variants that can grow in the presence of guanidine arise at a frequency of 10^{-5} to 10^{-6} (Cooper, 1968; Romanova et al., 1980). These viruses, upon isolation, exhibit phenotypes ranging from resistance to guanidine (gua^R) to dependence upon guanidine for growth (gua^D). Viruses that are resistant to guanidine sometimes complement wild-type guanidine sensitive viruses poorly (Ikegami et al., 1964), although the 2C region of the genome is highly conserved at the amino acid level among picornaviruses, suggesting that a required polypeptide function is encoded therein. The apparent lack of action in trans is not unique to this poliovirus protein; several viral polypeptides, most of them required very early in viral infection, are also not able to be complemented (Bernstein, et al., 1986). Furthermore, the degree of complementation between 2C alleles appears to be allele-specific (Ikegami et al., 1964).

Selection and Characterization of 3NC-202 gua^R Virus

When temperature sensitive 3NC-202 virus was grown under agar containing 0.5 mM guanidine, variants that could form plaques in the presence of guanidine appeared at a frequency of 5×10^{-6} (Table 1). These plaques represented guanidine-dependent virus; one isolate, 3NC-202gua^D, formed 200-fold more plaques in the presence than in the absence of 0.5 mM guanidine (Table 1). When plaques formed in the absence of guanidine were picked, one isolate was found to be truly guanidine resistant. This virus, designated 3NC-202gua^R, formed identical numbers of plaques in the presence of 0.5 mM guanidine and its absence. This gua^R allele also conferred an additional temperature sensitivity to the 3NC-202gua^R, virus; the reversion frequency at high temperature of 3NC-202gua^R virus was 10^{-4} lower than that of the 3NC-202 temperature sensitive virus alone (Table 1). The simplest explanation of these observations is that the mutation responsible for converting the gua^D allele to the gua^R allele also conferred additional temperature sensitivity; sequence and genetic data to support this idea will be presented below. Variants of foot and mouth disease virus, another picornavirus, have been observed to acquire temperature sensitive characteristics in conjunction with a guanidine resistant phenotype (Saunders and King, 1982).
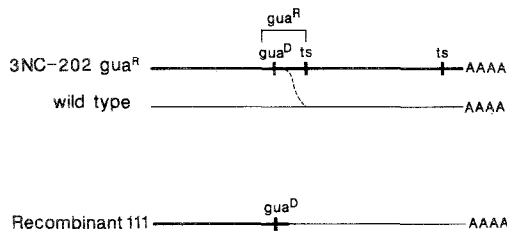
Figure 1. Diagram of Recombinant Cross

Positive strands of poliovirus genomes are shown as lines terminating in poly(A) at their 3' ends; sites of nucleotide changes in *3NC-202gua*^{R} and guanidine-dependent intratypic recombinant R111 viruses (Table 1) are denoted by slashes. The dotted line indicates the location of recombination events capable of leading to the formation of progeny viruses that would be able to grow at 39°C in the presence of 0.5 mM guanidine.

The sequence changes correlating with the guanidine dependent, guanidine resistant, and temperature sensitive phenotypes of the 2C alleles used in the present study are shown in Table 1. Sequencing of the wild type, *3NC-202gua*^{R}, and intertypic recombinant R111 genomes throughout the regions of the genomes encoding protein 2C was performed by limited chemical degradation (Maxam and Gilbert, 1980) of cDNA made by reverse transcriptase-catalyzed elongation of 5'-end-labeled DNA oligonucleotide primers (see Experimental Procedures). One point mutation, changing methionine #187 to a leucine, correlates with the guanidine dependent phenotype. Recent sequencing studies have shown that two other isolates of guanidine dependent poliovirus variants bear two point mutations each, one of which is the same methionine to leucine change at position #187 (Pincus et al., 1986). For this reason, and because of the low frequency with which *gua*^{D} mutants are obtained, it is possible that there is another sequence alteration responsible for the *gua*^{D} phenotype. Extensive sequencing of the region of the genome encoding protein 2C has not revealed such a change; it has been observed previously that determinants of guanidine resistance in some alleles lie outside the 2C region (Pincus et al., 1986).

The *gua*^{R} RNA contains a second mutation that

changes valine #250 to alanine (Table 1). This alteration is separated from the position #187 change in recombinants described below and can, through these recombinants, be shown to correlate with the conversion of the *gua*^{D} allele to the *gua*^{R}, temperature sensitive allele of protein 2C. Thus, virus containing only the position #187 change bears a *gua*^{D} allele, while virus containing both changes bears a *gua*^{R} allele that also confers a temperature sensitive phenotype on the virus.

Recombinant Crosses

When the *3NC-202gua*^{R} virus described in the previous section and wild-type (guanidine-sensitive) virus coinfect the same cells at high multiplicities of infection, recombination between the two RNA genomes occurs. If the recombination event takes place between the *gua*^{D} and the temperature sensitive components of the *gua*^{R} allele, recombinant virus bearing the *gua*^{D} allele of the *3NC-202gua*^{R} parent and 3' sequences from the wild-type parent will be formed (Figure 1). Having lost both temperature sensitive components of the *3NC-202gua*^{R} parents, these recombinant viruses will be the only progeny capable of forming plaques at 39°C in the presence of guanidine, and thus can be easily selected.

Crosses were performed by coinfecting with *3NC-202gua*^{R} virus and wild-type virus under conditions permissive for both. Progeny virus harvested after 4 to 6 hr was analyzed by plaque assay to determine the individual yields of each parental virus and of the recombinants; from these data recombination frequencies were calculated for both intratypic and intertypic crosses. Parallel infections of each parental virus alone were also analyzed as controls (Table 2; Figure 2).

In intratypic crosses, cells were coinfected with type 1 *3NC-202gua*^{R} virus and a wild-type virus derived from the unmutagenized Mahoney type 1 cDNA clone (Racaniello and Baltimore, 1981b). In this mixed infection (line 3, Table 2), the yields of the parental viruses were roughly similar to each other and to their respective yields in single infections (Table 2, lines 1 and 2). However, recombinant progeny were only seen following coinfection with both *3NC-202gua*^{R} and its wild-type parents (Table 2).

The phenotypes of the recombinant viruses recovered

Table 2. Intratypic and Intertypic Recombinant Crosses between *3NC-202gua*^{R} and Wild-Type Poliovirus

| Cross | Infection | Yields | | | |
		Wild Type × 10⁵	3NC-202guaR × 10⁵	Recombinant × 10³	Recombination Frequency × 10⁻⁶
Intratypic	1. Wild type 1	370	(0.001)	(0.05)	(1.4)
	2. *3NC-202gua*^{R}	(0.004)	210	(0.05)	(2.3)
	3. Wild type 1 plus *3NC-202gua*^{R}	780	140	120	1300
Intertypic	4. Wild type 2	420	(0.02)	(0.05)	(1.1)
	5. *3NC-202gua*^{R}	(0.004)	210	(0.05)	(2.3)
	6. Wild type 2 plus *3NC-202gua*^{R}	790	55	0.65	7.6

The infection of HeLa monolayers, preparation of virus stocks and titering of progeny virus are described in Experimental Procedures and in the text. Yields of various viruses are given as plaque forming units/ml of virus stock; the infected cells were incubated at 32°C (permissive conditions for both parental viruses). Recombination frequency is given as the yield of recombinant virus divided by the sum of the yields of the parental viruses. Background resulting from leakage and reversion of the markers can easily be identified phenotypically. For this reason, the numbers of these kinds of plaques, and the predicted "recombination frequencies" resulting from this background, are shown in parentheses.
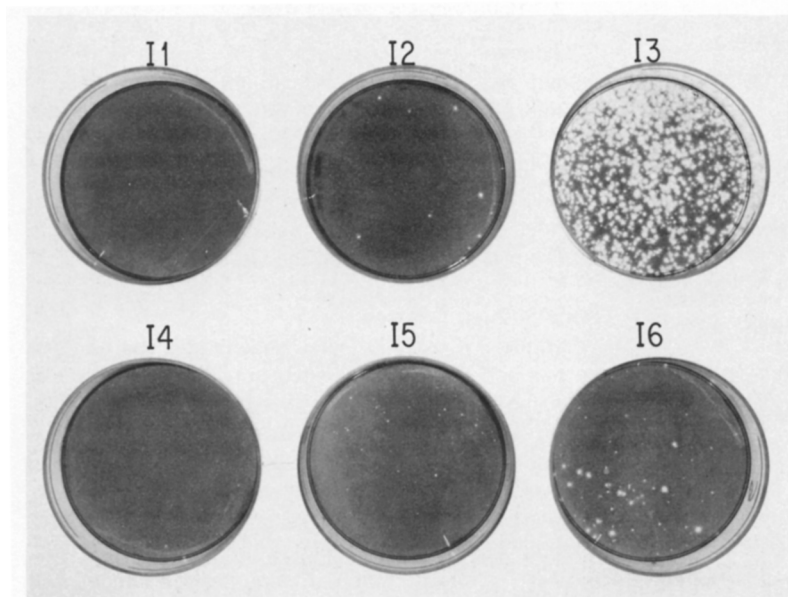
Figure 2. Plaque Assays under Nonpermissive Conditions of Intratypic and Intertypic Recombinant Crosses

HeLa monolayers that were infected with approximately 1:100 dilutions of virus stocks 1–6 (Table 2) are shown after incubation at 39°C for 48 hr under agar containing 0.5 mM guanidine. Infection 1 was wild-type, serotype 1 virus alone; Infection 2, $3NC$-$202gua^R$ virus alone; Infection 3; wild-type serotype 1 and $3NC$-$202gua^R$ viruses; Infection 4, wild-type serotype 2 virus alone; Infection 5, $3NC$-$202gua^R$ virus alone; and Infection 6, wild-type serotype 2 and $3NC$-$202gua^R$ viruses.

from intratypic crosses such as Infection 3 (Table 2) are typified by R111 (Table 1). Its guanidine dependent phenotype supports the idea that recombination in this cross occurred between the gua^D and temperature sensitive components of the gua^R allele of $3NC$-$202gua^R$ (Figure 1). Furthermore, the nucleotide sequence of R111 revealed that it did not contain the GTC (valine) to GCC (alanine) change at amino acid #250 of protein 2C (Table 1) apparently responsible for the temperature sensitive phenotype of the gua^R allele. Thus, the calculated recombination frequency of 1.3 × 10^{-3} recombinants (Table 2) in this cross reflected the frequency of recombination within a region of only 190 nucleotides. This point will be substantiated further by sequence analysis of intertypic recombinants between the same genetic markers.

Intertypic crosses were performed by coinfecting cells with $3NC$-$202gua^R$ virus and wild-type virus of Lansing serotype 2, derived from an infectious cDNA clone constructed by V. Racaniello (1985). Mahoney type 1 and Lansing type 2 poliovirus genomes share 85% overall homology on the nucleotide level (La Monica et al., 1986). As in the intratypic cross, an increase in plaque forming units under nonpermissive conditions resulted from mixed infection of the two parental viruses. The ease of detection of both intratypic and intertypic recombinants in this cross was apparent upon inspection of the results of plating virus stocks from Infections 1–6 (Table 2) under nonpermissive conditions (Figure 2). Although small plaques resulting from spontaneous mutation of the wild-type parental genomes were visible, recombinant progeny could be distinguished clearly in both crosses (Infections 3 and 6) by the larger size of the recombinant plaques as well as their increased frequency. Thus, both intratypic and intertypic recombination could be observed using these genetic markers; the frequency of intratypic recombination was more than 100 times higher than in the intertypic cross.

Sequence Analysis of Intertypic Recombinants

The occurrence of intertypic recombination between $3NC$-$202gua^R$ type 1 virus and wild-type 2 virus and the 15% sequence difference between the two viral serotypes made it possible to determine the locations of these intertypic genetic crossovers. In this way, the physical occurrence of RNA recombination in this system could be demonstrated, and the site specificity of RNA recombination investigated.

Individual intertypic recombinant plaques were picked from several different dishes such as plate I6 in Figure 2, representing progeny from several different recombinant crosses. These viral isolates were plaque-purified again, and their recombinant phenotypes were confirmed by plaque assay (data not shown). Thirteen of these intertypic recombinant viruses were further propagated, and individual RNA samples were prepared as described in Experimental Procedures.

To localize the crossover sites, RNA samples from recombinant and parental viruses were analyzed for their ability to protect complementary SP6 RNA probes from digestion by RNAases A and T1 (Zinn et al., 1983). The application of this method to the analysis of intertypic recombinants is diagrammed in Figure 3a. The RNA derived from each intertypic recombinant should be completely homologous at its 5′ end to the SP6 probes, which were transcribed from plasmids constructed from the Mahoney poliovirus serotype 1 cDNA. In a direction 3′ of the crossover junction, recombinant RNA should contain sequences derived from the serotype 2 parent, and should therefore display limited noncomplementarity to the type 1-derived probe. The crossover junction could be detected because RNA duplexes with noncomplementary regions as small as one nucleotide pair can be cleaved at the mismatched site by RNAase A (Winter et al., 1985; R. Myers and K. Zinn, personal communication).
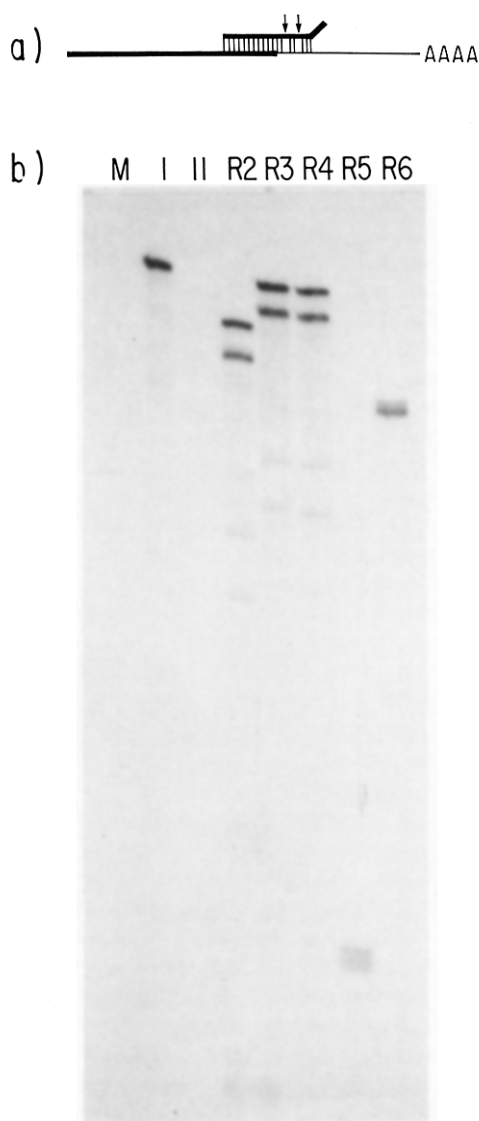
a)

b)

Figure 3. SP6 Mapping of Crossover Sites of Intertypic Recombinants

(a) The genome of an intertypic recombinant virus is shown schematically. Because of the method of selection for recombinants, the 5' half of the genome is expected to be derived from the serotype 1, *3NC-202gua*[R] parent, and the 3' half of the recombinant should be derived from the wild-type, serotype 2 parent. A uniformly labeled RNA transcript complementary to the serotype 1 parent should be protected from RNAase digestion throughout the length of the complementary region (Zinn et al., 1983); when the recombinant genome becomes identical with that of its type 2 parent, single base mismatches between the annealed RNAs are formed that can be cleaved by RNAase A, as indicated by arrows. (b) An autoradiogram of a sequencing gel displays the fragments of labeled RNA that were protected from RNAase digestion by hybridization with different RNA samples. From left to right, labeled RNA complementary to Mahoney serotype 1 poliovirus RNA from nucleotides 4600 to 4886 was annealed to RNA prepared from mock-, Mahoney serotype 1-, and Lansing type 2-infected HeLa cells, and cells infected with 5 isolates of intertypic recombinant viruses. R2 and R3 may have been siblings, as they were progeny of the same mixed infection. The preparation of cytoplasmic RNA and details of the SP6 mapping procedure are described in Experimental Procedures.

SP6 RNA analysis of several recombinants is shown in Figure 3b. The SP6 transcript used as a probe in this experiment contained nucleotides complementary to bases 4660 to 4886, in the region of the poliovirus genome encoding protein 2C. No protection of the SP6 probe was provided by RNAs prepared from mock- or type 2-infected HeLa cells, while a single protected band was seen with RNA prepared from type 1-infected cells. Each of the recombinant RNAs protected a smaller band, or set of bands, from RNAase digestion. The sizes of these bands established the approximate location of the crossover sites; pairs of bands presumably represent alternative partial cutting sites. The crossover junctions for all 13 intertypic recombinants were located within the sequences represented by the SP6 transcript shown in Figure 3. SP6 transcripts complementary to genomic regions 5' of these nucleotides (Experimental Procedures) were totally protected by RNA from all 13 recombinants and parental serotype 1 virus; SP6 transcripts complementary to genomic regions 3' of the recombination junctions (Experimental Procedures) were not protected by RNA from any of the 13 recombinants, yielding patterns indistinguishable from the serotype 2 parental viral RNA (data not shown). Thus, each of the 13 recombinants appeared from SP6 mapping to have been generated by a single recombination event.

The nucleotide sequences through the crossover regions of the 13 intertypic recombinant RNAs were determined by reverse transcriptase-catalyzed extension of synthetic deoxyoligonucleotide primers in the presence of dideoxynucleotides (Experimental Procedures). The nucleotide sequences of the parental viruses throughout the region in which recombination occurred are shown in Figure 4. The top line consists of the sequence of the *3NC-202gua*[R] Mahoney serotype 1 parent; small dots mark the locations of sequence differences between wild-type Mahoney type 1 virus (Racaniello and Baltimore, 1981b; Kitamura et al., 1981) and the *3NC-202gua*[R] mutant (Table 1). The bottom line shows the nucleotide sequence of the wild-type Lansing serotype 2 parent (La Monica et al., 1986). Sequence differences between the two parental viruses are marked with boxes. Each recombinant virus was found to be identical with the *3NC-202gua*[R] type 1 parent (Figure 4, top line) at its 5' end; within the marked regions the sequences of the numbered recombinant changed to that of the wild-type 2 parent. Due to the sequence homology between the two serotypes, only in the case of one intertypic recombinant (R3) could the exact pair of nucleotides flanking the crossover site be determined. All of the 13 intertypic recombinant sequences resulted from simple juxtaposition of the sequences of the two parental viruses.

Sequence and Structural Specificity of RNA Recombination

Several points can be made from the sequence locations of the crossover sites of the 13 intertypic recombinants (Figure 4). First, all of the recombinant junctions fell within the 190 nucleotides between the presumed *gua*[D] and temperature sensitive components of the *gua*[R] allele,
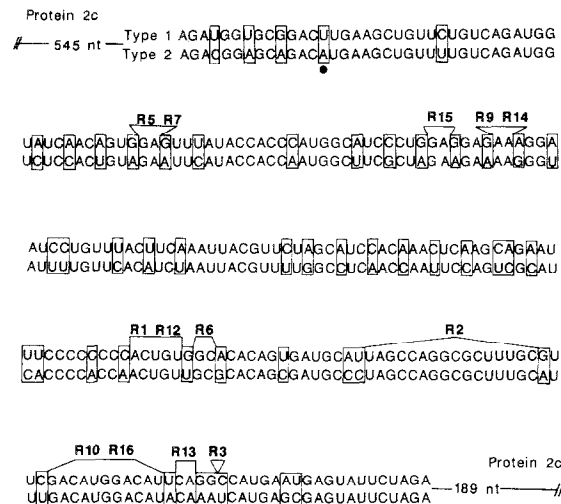
Protein 2c

Figure 4. Sequences at Recombinant Junctions
Crossover sites of 13 nonsibling intertypic recombinants were determined by reverse transcriptase-catalyzed extension of synthetic deoxyoligonucleotides in the presence of dideoxynucleotides (Experimental Procedures). The top line shows the sequence of the 3NC-202gua^R serotype 1 parent throughout the genomic region in which the crossover events occurred; the nucleotide differences between the 3NC-202gua^R virus (Table 1) and wild-type Mahoney serotype 1 poliovirus (Racaniello and Baltimore, 1981b) are marked by dots; in both cases the type 2 nucleotide is that found in the wild-type serotype 1. The bottom line shows the sequence of the wild-type Lansing serotype 2 parent (La Monica et al., 1986) throughout this region. Nucleotides that differ between the two parental viruses are boxed. All intertypic recombinant genomes were derived from the type 1 parent at their 5′ ends; the sequence of each recombinant changed to that of the serotype 2 parent at the indicated location.

supporting the model shown in Figure 1 for the composition of the gua^R allele. Second, within these 190 nucleotides, no striking sequence specificity of RNA recombination was observed. Finally, there appeared to be no preference for RNA recombination to occur in regions of great homology between the two parental viruses. In fact, the crossover site for one intertypic recombinant (R3) lay, somewhat improbably, in the least conserved sequence of the 190 nucleotide region.

Previous work had suggested that homology might be an important determinant of recombination rates. In one study, in which the crossover site of a recombinant poliovirus was sequenced, the virus was isolated from a vaccine-associated case of paralytic poliomyelitis and was found to result from recombination between polioviruses of vaccine serotypes 1 and 2 (Kew and Nottay, 1984). The junction between the type 1 and type 2 sequences lay in an 18 base region of homology between the two viral serotypes. Also, it has been reported that the frequencies of intertypic recombination between polioviruses in tissue culture are consistently lower than the frequencies of recombination between two viruses of the same serotype, albeit using different genetic markers (Tolskaya et al., 1983; McCahon et al., 1975). On the basis of these observations, a requirement for homology between parental viruses for RNA recombination to occur has been proposed (Tolskaya et al., 1983; McCahon et al., 1975).

We also observed that the frequency of intertypic recombination was more than 100 times lower than that of intratypic recombination, in this case using the same genetic markers in the two kinds of crosses (Table 2). The lower frequency of intertypic recombination could possibly reflect a lack of viability of many intertypic recombinants. Throughout the 190 nucleotides in which the crossover events occur, only 3 of the 39 nucleotide changes between serotypes 1 and 2 predict amino acid changes in protein 2C. Nevertheless, the plaque phenotypes of the 13 intertypic recombinants, while all relatively guanidine-dependent and temperature-insensitive, vary significantly and unpredictably. It is possible that single nucleotide changes in other regions of the genome, not evident from the SP6 probe analysis perhaps because they are in the type 2 region, may have improved the otherwise poor viability of the intertypic recombinants, modifying their phenotype. A requirement for such nucleotide changes would explain a much lower frequency of intertypic recombination in this region. Nonetheless, it is also likely that the lower intertypic recombination frequency reflects some preference for homology between recombining parents for recombination to occur. No addition or deletion of bases was seen for any of the intertypic recombinants, arguing, perhaps, that there is some alignment of the parental molecules. It is clear from Figure 4 that identity between the two parental RNA molecules immediately at the crossover junction does not seem to be necessary for RNA recombination. Thus, if there is a role for overall homology between the two parental genomes, it may result from a need for a general alignment of the parental molecules or for stabilization of some structure larger than that immediately at the crossover site.

These findings appear to eliminate certain classes of models for the mechanism of RNA recombination. For example, all autocatalytic RNA processing reactions reported thus far have been extremely site-specific (Cech and Bass, 1986). Breaking and rejoining models employing specific endonucleases, as well as copy choice mechanisms in which the viral RNA polymerase switches strands at specific sequence signals, as proposed for the generation of defective interfering particles in negative strand viruses (for example, Lazzarini et al., 1981; Fields and Winter, 1982), are inconsistent with the low level of sequence specificity observed for poliovirus RNA recombination. Studying the sequences at which RNA recombination occurred, however, did not suggest a particular mechanism for its occurrence. For this, more direct tests of specific models were devised.

Breaking and Rejoining or Copy Choice Mechanism of RNA Recombination

The poliovirus mutants used as genetic markers in this work, the 3NC-202 temperature sensitive mutant and the guanidine resistant mutant, both affect viral replication. 3NC-202 has been shown to have a noncomplementable defect in negative strand synthesis at the nonpermissive temperature (Sarnow et al., 1986; P. Sarnow, K. Kirkegaard, and D. Baltimore, unpublished results; H. Bernstein, personal communication). Wild-type poliovirus replication

Table 3. Effects of Inhibiting Replication of Parental Viruses on Recombination Frequency

Experiment	Infection	Time at 32°C	Chase	Super-infection	Growth	Yields Wild Type × 10^5	3NC-202gua^R × 10^5	Recombinants × 10^3	Recombination Frequency × 10^{-6}
Simple coinfection	1. Wild type	4 hr		None		450	(0.0005)	(0.15)	(0.3)
	2. 3NC-202gua^R	4 hr		None		(0.0005)	600	(0.02)	(3.0)
	3. Wild type plus 3NC-202gua^R	4 hr		None		230	330	10	180
Inhibit 3NC202gua^R replication	4. None	4 hr	0.5 hr 39°C	Wild type	2.5 hr 39°C	130	(0.0005)	(0.05)	(4.0)
	5. 3NC-202gua^R	4 hr	0.5 hr 39°C	None	2.5 hr 39°C	(0.0005)	150	(0.02)	(2.0)
	6. 3NC-202gua^R	4 hr	0.5 hr 39°C	Wild type	2.5 hr 39°C	32	75	4.0	380
Inhibit wild-type replication	7. Wild type	5 hr	0.5 hr 0.5 mM gua	None	5 hr 0.5 mM gua	280	(0.0005)	(0.30)	(1.0)
	8. None	5 hr	0.5 hr 0.5 mM gua	3NC-202gua^R	5 hr 0.5 mM gua	(0.0005)	85	(0.02)	(2.0)
	9. Wild type	5 hr	0.5 hr 0.5 mM gua	3NC-202gua^H	5 hr 0.5 mM gua	170	95	(0.10)	(0.4)

In Infections 1–3, monolayers of HeLa cells were infected with the parental viruses individually and in combination, and allowed to grow for 4 hr at 32°C. Progeny were harvested and titered as described in Experimental Procedures. In Infections 4–6, HeLa cells were first mock-infected or infected with 3NC-202gua^R virus and incubated under permissive conditions, 32°C. After 4 hr, the plates were placed at 39°C for 30 min to inhibit replication of the 3NC-202gua^R virus. The monolayers were then superinfected with wild-type 1 virus or were mock-infected and incubated at 39°C for 2.5 hr before progeny were harvested and assayed. In Infections 7–9, monolayers of HeLa cells were first infected with wild-type 1 virus or were mock-infected and incubated at 32°C for 5 hr before replication of the wild-type virus was inhibited by adding new medium, containing 0.5 mM guanidine to the dishes. After 30 min the cells were superinfected with 3NC-202gua^R virus or were mock-infected and incubated for 5 hr at 32°C in the presence of 0.5 mM guanidine. Yields of each virus are expressed as plaque forming units/ml of virus stock at the appropriate assay condition (Experimental Procedures).

is inhibited by guanidine; synthesis of both negative and positive strands is affected (unpublished observations). Coinfection of wild-type guanidine sensitive virus with virus bearing the guanidine resistance allele used in this study does not alleviate this inhibition (unpublished results, Table 3). Thus, the replication of the 3NC-202gua^R temperature sensitive parental virus and the wild-type guanidine sensitive parental virus could be inhibited independently of each other in recombinant crosses, and the effect on the frequency of recombination could be determined.

Different models of the mechanism of RNA recombination predict different outcomes for this experiment, as diagrammed in Figure 5. If RNA recombination occurs by a breaking and joining mechanism, it is difficult to predict

whether ongoing replication would be required or not. One can assume, however, that the requirements for the two recombining parents would be symmetrical. For example, if only the physical presence and not the replication of the 3NC-202gua^R parental RNA were necessary for it to be a substrate for breaking and rejoining enzymes, it is difficult to imagine why the wild-type parental RNA would have to be replicating to provide a substrate for the same enzymes. Thus, the prediction of a breaking and rejoining model of RNA recombination is that inhibiting the replication of the 3NC-202gua^R virus with high temperature and inhibiting the replication of the wild-type virus with guanidine should have the same effect on the frequency of RNA recombination.

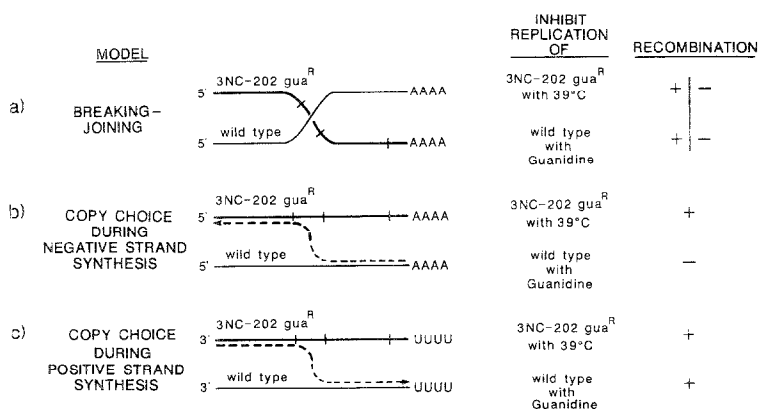If RNA recombination occurs by copy choice during

Figure 5. Predictions of Models of RNA Recombination on the Effect of Individually Inhibiting the Replication of the Parental Viruses

The heavy lines represent the positive (a and b) or negative (c) strands of the 3NC-202gua^R parental virus; slashes show the positions of the sequence differences from the wild-type genome (Table 1). The lighter lines represent the positive (a and b) and negative (c) strands of the wild-type parental virus in these crosses (Table 3). The recombination models and their implications are discussed in the text.

negative strand synthesis, a different result is expected. To generate a recombinant that possesses the ability to grow in the presence of guanidine but does not bear any temperature sensitive markers, the order of the markers on the genome dictates that synthesis of the recombinant negative strand must initiate at the 3' end of the wild-type viral RNA (Figure 5). This event, and therefore RNA recombination by this model, should be inhibited by guanidine but should be unaffected by high temperature.

To produce a selectable recombinant virus in this cross by copy choice during positive strand synthesis, synthesis of the recombinant plus strand must initiate at the 3' end of the 3NC-202gua^R negative strand (Figure 5). This parental virus is guanidine resistant, and therefore guanidine should have no effect on recombination by this mechanism. High temperature, which inhibits only negative strand synthesis of the 3NC-202gua^R virus, should also have no effect on the frequency of RNA recombination if it occurs by copy choice during positive strand synthesis.

To obtain a yield of each parental virus sufficient to participate in recombination by any mechanism, but still able to inhibit the RNA synthesis of one of the parental viruses during the coinfection, the following scheme was employed. Infection was carried out with one parent, followed by superinfection under nonpermissive conditions for the initially infecting parent, but using permissive conditions for the second parent (Table 3). Infections 1 to 3 were identical with those shown in Table 2, but with shorter incubation times. Infection 3 was a simple coinfection of wild-type 1 and 3NC-202gua^R viruses; recombinants arose at a frequency of 1.8 $\times$ 10^{-4}. Infection 6, on the other hand, was a coinfection in which wild-type virus was allowed to grow and replicate in the presence of a large pool of non-replicating 3NC-202gua^R virus. This was accomplished by first infecting HeLa cells with 3NC-202gua^R virus for 4 hr under permissive conditions, shifting to 39°C for 30 min to inhibit 3NC-202gua^R replication, superinfecting with wild-type virus at 39°C, and allowing the wild-type virus to grow at 39°C for 2.5 hr in the presence of the nonreplicating 3NC-202gua^R virus. As can be seen from Infection 6 in Table 3, and control Infections 4 and 5, this protocol yielded comparable titers of both parental viruses. Most important, the recombination frequency was similar to that resulting from a simple coinfection, showing that all parental viruses do not have to be replicating to serve as substrates for recombination.

In the reciprocal experiment, Infection 9, HeLa cells were infected with wild-type virus for 5 hr under permissive conditions, and then 0.5 mM guanidine hydrochloride was added to inhibit wild-type RNA synthesis. After 30 min, these cells were superinfected with 3NC-202gua^R virus, which was allowed to grow for 5 hr in the presence of 0.5 mM guanidine and the pool of nonreplicating wild-type virus. This infection, and control Infections 7 and 8, gave comparable yields of wild-type and 3NC-202gua^R virus. There were, however, no observable recombinant progeny resulting from Infection 9, corresponding to a recombination frequency 100 times lower than that observed in Infection 6. There exists a formal possibility that guanidine inhibits recombination for some other reason,

although it has been observed previously that guanidine stimulates recombination in some crosses (Cooper, 1968). Experiments designed to show that guanidine does not inhibit recombination, involving crosses between wild-type virus and a virus with a temperature sensitive marker 5' of the guanidine resistance locus, have been difficult because of the lack of availability of a characterized mutant with a sufficiently low reversion rate in the 5' half of the poliovirus genome. We interpret the data in Table 3 to mean that recombination did not occur in Infection 9 because replication of the wild-type virus was inhibited. We acknowledge, however, that we have not ruled out the formal possibility that guanidine directly inhibits recombination.

The finding that RNA recombination between 3NC-202gua^R and wild-type polioviruses could occur when replication of the parental virus donating the 5' end of the recombinant RNA was inhibited, but not when replication of the parental virus donating the 3' end was inhibited, is consistent only with Figure 5, model b. Thus, these data suggest that RNA recombination in poliovirus results from a template switch made by the viral polymerase during negative strand synthesis. The sequences of intertypic recombinants (Figure 4) suggested that RNA recombination is not particularly site-specific. Thus, the apparent preference of the polioviral replicase to switch templates during negative strand rather than positive strand synthesis presumably results from different structures or availabilities of the templates for negative and positive strand synthesis during viral replication. Support for this idea comes from the finding that most recombination events between foot-and-mouth disease mutants occur very early in the infectious cycle (McCahon and Slade, 1981).

Genetic recombination on the RNA level has been demonstrated in a murine coronavirus (Makino et al., 1986) and in a multipartite plant virus (Bujarski and Kaesberg, 1986), as well as in picornaviruses. Possibly related RNA rearrangements include the generation of defective interfering particles in positive and negative strand RNA viruses, the fusing of noncontiguous RNA sequences during coronavirus transcription (Baric et al., 1985), and the duplication of small segments of RNA in viroids (Keese and Symons, 1985). Copy choice, or "polymerase jumping" mechanisms, have been invoked to explain many of these phenomena. There are, however, only a few experimental indications to support this view. The most compelling of these indications comes from coronaviruses; UV mapping and pulse-chase experiments have suggested that coronavirus transcripts, most of which display specific internal deletions relative to the viral genome, do not originate from larger precursors, but instead are transcribed in a discontinuous manner (Jacobs et al., 1981; Stern and Sefton, 1982; Baric et al., 1985). That template switching of the polioviral RNA-dependent RNA polymerase is apparently responsible for RNA recombination in poliovirus strengthens the idea that other RNA rearrangements may occur by this mechanism as well, and it revives copy choice as a viable mechanism for some kinds of genetic rearrangements.

Experimental Procedures

Viruses

High titer stocks of the 3NC-202 temperature-sensitive poliovirus mutant (Sarnow et al., 1986) were a gift of Dr. P. Sarnow. Gua^D derivatives were obtained by infecting monolayers of HeLa cells, maintained in Dulbecco modified Eagle's medium (DME) supplemented with 10% fetal calf serum, with approximately 5×10^6 3NC-202 viruses per 60 mm dish. After 30 min adsorption in 0.2 ml of phosphate buffered saline (PBS), an overlay containing DME, 5% fetal calf serum, 1% agar, and 0.5 mM guanidine hydrochloride (Mallinckrodt) was applied. Plaques were picked after 72 hr of incubation at 32°C, and titered by plaque assay with and without 0.5 mM guanidine. Almost all of these plaques displayed some dependence on the presence of 0.5 mM guanidine for growth; several plaques that appeared in the plates lacking guanidine were isolated and reassayed. One isolate, 3NC-202gua^R, showed good growth characteristics (relatively large plaques and a low level of reversion during plaque assay) in the presence and absence of 0.5 mM guanidine. Single plaques of 3NC-202gua^R were isolated, and high titer virus stocks were prepared by infection of monolayers of HeLa cells.

Mahoney serotype 1 poliovirus was propagated from a single plaque, derived from a cDNA-containing plasmid as described (Racaniello and Baltimore, 1981b). DNA plasmid pVR201, containing a cDNA replica of the Lansing serotype 2 poliovirus genome, was kindly provided by Dr. V. Racaniello. Monolayers of HeLa cells were transfected using DEAE dextran (Sompayrak and Danna, 1981); single plaques were picked and expanded into high titer stocks by infection of HeLa monolayers.

Recombinant Crosses

In simple infections and coinfections, monolayers of HeLa cells in 60 mM petri dishes were washed once with PBS, and viruses were added in the appropriate combinations and multiplicities of infection (moi). Unless stated differently, the wild-type polioviruses were present at moi of 10, and the 3NC-202gua^R poliovirus at an moi of 50. After 30 min at 32°C, unadsorbed virus was removed by washing with PBS. DME containing 10% fetal calf serum was added, and plates were incubated at 32°C for 4–6 hr. Incubation at 32°C, with no guanidine in the medium, provided a permissive growth condition for both parental viruses. When a primary infection was followed by superinfection with a second virus, times of adsorption for both viruses were reduced to 20 min, and the second incubation condition was nonpermissive for the virus that was used in the first infection.

After incubation, cells were washed, resuspended in 1 ml of PBS, and lysed by freezing and thawing. Nuclei and cell debris were removed by centrifugation at $1500 \times g$ for 10 min at 4°C, and the resulting virus stocks were titered under the following three conditions: at 32°C, with 0.5 mM guanidine, to determine the yield of the 3NC-202gua^R parent; at 39°C, with no guanidine, to determine the yield of the wild-type serotype 1 or serotype 2 parent; and at 39°C with 0.5 mM guanidine, to determine the yield of recombinants and the amount of background from spontaneous mutations. Infected monolayers were incubated under agar for 48 and 72 hr for 39°C and 32°C infections, respectively, before the agar was removed, and the monolayers were stained with a solution containing 20% ethanol and 0.1% crystal violet (Fluka AG). Yields of the different viruses are expressed as plaque forming units (PFU) per ml, as assayed under the conditions appropriate for each virus. Recombinant crosses were repeated many times; examples are presented in Table 2 and Table 3.

Individual intertypic recombinant plaques were picked from plaque assays incubated at 39°C in the presence of 0.5 mM guanidine, plaque-purified a second time, and propagated on monolayers of HeLa cells.

Preparation of Cytoplasmic RNA

Pure stocks of Mahoney type 1, Lansing type 2, 3NC-202gua^R, intratypic recombinant R111, and intertypic recombinants R2, R3, R5, R6, R7, R9, R10, R11, R12, R13, R15, and R16 were used to infect ten 100 mm plates of HeLa cells, each at an moi greater than 5. Infected monolayers were incubated at 39°C in DME containing 10% fetal calf serum and 0.5 mM guanidine until cytopathic effects developed, whereupon cells from each infection were harvested by scraping, pooled, resuspended in 10 ml of buffer containing 10 mM Tris (pH 7.8),

1 mM EDTA, and 150 mM NaCl, and lysed by freezing and thawing. Nuclei and cell debris were removed by centrifugation, and the supernatant was made 0.5% in sodium dodecylsulfate. After two phenol extractions, the supernatant was transferred to autoclaved 25×89 mm polyallomer centrifuge tubes (Beckman); 1.0 ml of 3.0 M sodium acetate and 25 ml of ethanol were added, and the solution was frozen on dry ice for 30 min. The precipitated RNA was collected by centrifugation at 15,000 rpm for 15 min in an SW28 rotor (Beckman) and was then redissolved in 0.9 ml of water. Sodium acetate was added to a final concentration of 0.3 M; 3 ml of ethanol was added, and 0.4 ml aliquots were apportioned into individual 1.5 ml tubes (Eppendorf). These tubes were stored at $-20°C$; one aliquot of cytoplasmic RNA, after precipitation, was usually sufficient for SP6 mapping or any of the sequencing protocols employed.

Sequencing and SP6 Mapping

The sequence of the 3NC-202gua^R virus throughout the genomic region 2C was determined by base-specific chemical cleavage (Maxam and Gilbert, 1980) of cDNA made by reverse transcriptase-catalyzed extension of synthetic oligonucleotides, labeled with ^{32}P at their 5' ends (Reilly et al., 1984). The oligonucleotides used were GGTTTCCAG-GATTTC, complementary to nucleotides 4284 through 4299 in the poliovirus genome, AGTATGCTCTAGTTTC (4431 through 4446), CAC-CACTCCCTGTTG (4629 through 4643), GATTCCTTTCTCCTCC (4742 through 4757), ACACATTTCAGTAGC (4917 through 4931), and ATA-CTGGAGTGGTCC (5112 through 5126); they were synthesized by C. Riser and W. Brown (Whitehead Institute).

SP6 mapping was performed according to Zinn et al. (1983), under the following conditions: cytoplasmic RNA from mock-infected or poliovirus-infected cells was hybridized with ^{32}P-labeled SP6 transcripts at 60°C; the RNAase A- and RNAase T1-containing buffer was 100 mM in NaCl, and the RNA hybrids were treated with the RNAase solution for 10 min at 15°C. These digestion conditions appear to optimize the recognition of single base mismatches by RNAase A (data not shown; R. Myers, personal communication). Individual SP6 transcripts complementary to nucleotides 3581 to 4886, 4886 to 6304, 6304 to 7387, and 4600 to 4886 of the Mahoney type 1 poliovirus genome were used to analyze the recombinant genomes.

The crossover sites of the intertypic recombinants were sequenced by dideoxynucleotide chain termination (Sanger et al., 1977) essentially as described by Emini et al. (1984), except that the reverse transcriptase (BRL) reactions were terminated by the addition of 150 μl of a solution containing 1.5 M ammonium acetate, 10 μg/ml of tRNA, and 0.5 ml of ethanol. The solutions were then frozen; the RNA was collected by centrifugation and rinsed with cold 80% ethanol before being loaded on a sequencing gel. After electrophoresis, the gel was visualized by autoradiography without further treatment. The primers for dideoxynucleotide sequencing of the 13 recombinants were synthetic deoxyoligonucleotides CACATTTCAGTTGCC, complementary to nucleotides 4912 through 4926, and GTTGAGGCCAAAACG, complementary to nucleotides 4772 through 4786 of the Lansing type 2 poliovirus genome (La Monica et al., 1986).

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