On the Nature of Poliovirus Genetic Recombinants

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SUMMARY

Recombinant and parental poliovirus particles were indistinguishable by rate-zonal and isopycnic sedimentation, and by u.v. inactivation. Sensitive selective procedures, applied to ts+ recombinants to detect genetic segregation of one parent, failed to reveal any. Poliovirus genetic recombinant particles thus appear to be conventional virus particles; their significance for recombination mechanisms is discussed. Sensitivity to the growth inhibitor 2-(3-chloro-p-tolyl)-5-ethyl-1,3,4-oxadiazole is shown to depend on a product of the structural protein gene.

INTRODUCTION

Novel combinations of DNA genetic material may arise either by reassortment of large linkage groups (chromosomes) or by recombination within linkage groups, i.e. of DNA pieces covalently broken and rejoined by enzymes. With one known exception, 'recombination' of RNA genetic material (only found among viruses) either does not occur, or seems to arise by simple reassortment of unlinked pieces of RNA analogous to very small chromosomes ('segmented genomes').

Picornaviruses provide the unique exception. A phenomenon with the appearance of genetic recombination is well established for these viruses, but their genome is not naturally segmented and there are strong theoretical reasons to expect that the recombinant RNA is also in a single covalently joined piece. However, no experimental evidence has been available on this point.

This paper examines some properties of poliovirus genetic recombinants for their relevance to the mechanism of recombination in this ribovirus. The results argue in favour of a precisely ordered molecular exchange similar to that of DNA systems, but the question of breakage–reunion versus copy–choice remains open.

METHODS

Virus strains and assays and the standard recombination test have been described previously (Cooper, 1968). The e.o.p. of a virus in presence of an inhibitor compound equals the number of plaques formed when the drug is incorporated in the agar of plaque assay plates divided by the number of plaques formed in the absence of the drug. The ts-3 × 28g recombinant pool described below (crude culture fluid) was centrifuged for 30 min at 7000 g and the

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supernatant fluid used for the physical analyses. For the sucrose gradients, the virus particles in the clarified recombinant pool were sedimented (2 h at 100,000 g), resuspended in 1/6 vol. 0.14 M-NaCl, and centrifuged through a 15°5 ml linear 15 to 30% (w/w) sucrose gradient in 0.3 M-NaCl, 0.03 M-sodium citrate, pH7, 0.01 M-EDTA (3.3 h at 70,000 g in a Spinco SW25.3 rotor at 4°C). For isopycnic sedimentation, 1 ml of the clarified recombinant pool was diluted with 9 ml of 0.01 M-tris HCl (pH 7.4), 0.01 M-KCl, 0.0015 M-MgCl₂, the mixture used to dissolve 5 g CsCl, and the solution then centrifuged for 24 h at 110,000 g (Spinco 40 rotor). Gradients were fractionated from a hole in the bottom of the tube; CsCl density was determined by refractive index. For u.v. inactivation, 0.5 ml of the clarified recombinant pool was mixed with 0.5 ml of 0.5 M-glycine (pH 2.5) to dissociate clumps, and adjusted to pH 7 after 5 min at 18°C with 1.5 ml of 0.14 M-NaCl, 0.01 M-phosphate (pH about 8) containing 1% tryptose. This material was irradiated in an open 5 cm Petri plate on a tilted rotating platform 30 cm from a 15 W u.v. lamp; at intervals, 0.5 ml samples were removed to ice for assay. The ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate (S-7, Yamazi, Takahashi & Todome, 1970) was a generous gift from Toyama Kagaku Kogyo Co., Tokyo; the 2-(3-chloro-p-tolyl)-5-ethyl-1,3,4-oxadiazole was prepared by Eli Lilly & Co.

The sedimentation of recombinant RNA was examined by procedures described by Cooper, Staněk & Summers (1970a). A suspension of U cells was infected (5 p.f.u./cell) at 0°C, and incubated for 2.5 h in medium containing 0.5 μg actinomycin D/ml, when [3H]-uridine was added to 10 μCi/ml. Cultures were frozen after a further 3 h, debris removed (10 min centrifuging at 7000 g), the virus sedimented and banded in CsCl. Virtually all the label was in a band at 1.34 g/ml, which was diluted in PBS and the virus resedimented. It was then resuspended in NETS buffer (0.1 M-NaCl, 10 mM-tris HCl, pH 7.4, 10 mM-EDTA, 5 mg/ml SDS), heated at 60°C for 5 min, and the liberated RNA centrifuged through 15 to 30% sucrose gradients in NETS for 17 h at 42,000 g in the Spinco SW 27 rotor at 25°C.

RESULTS

Physical identity of recombinant and parental poliovirus particles

The well-characterized cross ts-3 × 28g was used for the following experiments because it covers about half the genetic map, involves two independent gene functions (Cooper, 1969; Cooper et al. 1971) and yet has a low content of background revertants. The standard recombination test (Cooper, 1968) was used to provide a 12 ml pool of progeny from the cross ts-3 × 28g with an accurately assayed proportion of ts⁺ recombinants: in 19 assays, the mean frequency ± standard error was 0.374 ± 0.057% after subtraction of revertant content. This is within the normal range for this pair, but as extensively considered previously (Cooper, 1968), and as seen in Fig. 1, the absolute values can vary over about a twofold range between assays. Relative values within an assay series remain constant. The ts⁺ revertant content calculated from concurrent self-crosses was 0.019%, i.e. 1/20 of the total ts⁺ content, and so for practical purposes the recombinant content was taken as equal to the ts⁺ content.

This recombinant pool was analysed by rate-zonal sedimentation through linear sucrose gradients, by isopycnic sedimentation in CsCl, and by u.v. inactivation (see Methods). Fractions were plaque-assayed at 37°C and 39.5°C after dilution through pH 2-5 buffer as previously described (Cooper, 1968). Each analysis was done twice, with the same result: the tests failed to distinguish between recombinant and parental types in sedimentation coefficient (Fig. 1A), density in CsCl (Fig. 1B) and in u.v. target size (Fig. 1C). The u.v. inactivation of the recombinants was first order to 10⁻² survivors, so that the target was not
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Fig. 1. Analysis of the ts+ content of progeny from the cross ts-3 × 28g by three physical tests: A, rate-zonal sucrose gradient sedimentation; B, equilibrium density sedimentation in CsCl; C, u.v. inactivation (2 experiments, squares and triangles). Experimental details are given in Methods; the tops of gradients are at the right. Open symbols at the top refer to the percentage ts+ contents of those fractions that contained sufficient ts+ for assay; open symbols in the lower parts refer to results of plaque assays at 37 °C, filled symbols to results of plaque assays at 39.5 °C.

multiple in nature, and an insignificant proportion comprised a smaller or larger target than that of the parental virus. This also provides good evidence that the proportion of clumps was negligible. All three methods should distinguish RNA contents that differed by 10 to 15%, particularly the CsCl gradients.

Lack of genetic segregation of parental phenotype from recombinant poliovirus clones

Preliminary tests showed that gross segregation of parental ts phenotypes from poliovirus ts+ recombinants did not occur. A small amount of segregation would be difficult to establish, however, as ts+ stocks may spontaneously contain 0.1 to 1% of ts mutants (Cooper, 1964). This difficulty was overcome by exploiting the resistance of ts-28g to guanidine, and of two other ts mutants to two inhibitors of ts+, which are described below. The two mutants that are resistant to the inhibitors were detected by testing a range of ts mutants against a range of inhibitors (unpublished results). The theoretical basis for these tests is that a hypothetical heterozygote (isolated as a ts+ 'recombinant' because it could grow at restrictive temperature by genetic complementation) would be expected to yield some segregants of the parental phenotype. If one of the parents was resistant to an inhibitor, then the presence of a heterozygote should be revealed either by an inhibitor-resistant ts+ 'recombinant' or by segregation, i.e. the appearance of a parental, inhibitor-resistant phenotype in the progeny. Thus these inhibitors should provide a sensitive selective procedure to detect genetic segregation in these instances.

The mutants used were ts-28g (resistant to guanidine), ts-2 (one of several structural-protein mutants co-variantly resistant to growth inhibition by ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate, or S-7 (Cooper et al. 1971)), and ts-3, another structural-protein mutant co-variantly resistant to 2-(3-chloro-p-tolyl)-5-ethyl-1,3,4-oxadiazole. The resistance of ts-3 to the oxadiazole derivative is illustrated in Fig. 2.

Stocks for testing genetic segregation were made with standard recombination tests using
Fig. 2. The sensitivity of poliovirus strains to 2-(3-chloro-p-tolyl)-5-ethyl-1,3,4-oxadiazole, at the given concentrations in plaque assay medium. Left: distribution of efficiencies of plating (10 μg of oxadiazole derivative/ml:zero) at 37 °C of 19 ts-3 x 20 recombinants, ts+, ts-3 and ts-20, and 11 ts+ revertants from ts-3. Right: plaque formation at 37 °C in various concentrations of oxadiazole derivative by ts+, ts-20 and ts-3.

the crosses ts-3 x 28g, ts-3 x 20, and ts-2 x 20. Mutants ts-20 and ts-28 are in the left-hand half of the genetic map and are defective in non-structural functions, while ts-2 and -3 are in the right-hand half and are defective in structural protein genes. The g (guanidine resistance) locus lies in the structural protein region. The 12 ml progeny pool described above was used for the cross ts-3 x 28g. In the progeny pool obtained from the cross ts-3 x 20, the recombination frequency was 0.1%, rather low for this pair but 12 times the spontaneous reversion expected from concurrent self crosses. In the progeny pool obtained from the cross ts-2 x 20, the recombination frequency was 0.6%, normal for this pair and 10 times the background. Thus at least 90% of ts+ clones picked from all three crosses will be recombinant rather than revertant. Wild-type plaques picked from 39.5 °C assays of these crosses were incubated at 37 °C in tube cultures in absence of any inhibitor until c.p.e. was evident (2 days), giving titres of 0.7 to 4 × 10^7 p.f.u./ml, with e.o.p. (39.5 °C: 37 °C) all about 2. The results are summarized in Table 1, and discussed in the text below.

One plaque from the 37 °C assay of each of 29 ts+ clones from the ts-3 x 20 cross was passed again in tube cultures (2 days at 37 °C). All 29 reclones were as sensitive as ts+ to the oxadiazole derivative at concentrations up to 20 μg/ml. For example (Table 1), the e.o.p. (15:0 μg/ml) at 37 °C were 0.0021 (ts+), 0.002 (ts-20), 0.0005 to 0.0073 (recombinants; mean = 0.0021) and 0.42 (ts-3). The proportion of drug-resistant variants, which would include ts-3 segregants, will be less than the e.o.p., as this also includes some ‘leak’ of the sensitive
Table 1. Efficiencies of plating in various inhibitors of parental ts strains and recombinant ts+ clones from three poliovirus crosses

<table>
<thead>
<tr>
<th>Cross</th>
<th>Strains tested</th>
<th>e.o.p.</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts-3 × 20</td>
<td>Recombinants: 29 ts+ clones</td>
<td>0.0005 to 0.0073</td>
<td>Oxadiazole (15.0 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>Parents: ts-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>: ts-20</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild type: ts+</td>
<td>0.0021</td>
<td></td>
</tr>
<tr>
<td>ts-2 × 20</td>
<td>Recombinants: 24 ts+ clones</td>
<td>0.0012 to 0.01</td>
<td>S-7</td>
</tr>
<tr>
<td></td>
<td>Parents: ts-2</td>
<td></td>
<td>(2.5 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>: ts-20</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild type: ts+</td>
<td>0.0063</td>
<td></td>
</tr>
<tr>
<td>ts-3 × 28g</td>
<td>Recombinants: 9 ts+ clones</td>
<td>0.00001 to 0.0002</td>
<td>Guanidine carbonate (150:10 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>Parents: ts-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>: ts-28g</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild type: ts+</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Efficiencies of plating of clones selected from an assay in 2 μg S-7/ml at 37 °C of two ts+ recombinant clones from the cross ts-2 × 20

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cystine (0.01 mm: 0.1 mm)</th>
<th>S-7 (2 μg/ml: 0 μg/ml)</th>
<th>39.5 °C: 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-7 clone 1</td>
<td>2.1</td>
<td>50</td>
<td>0.043</td>
</tr>
<tr>
<td>S-7 clone 2</td>
<td>0.5</td>
<td>2.5</td>
<td>0.0007</td>
</tr>
<tr>
<td>S-7 clone 3</td>
<td>0.03</td>
<td>2</td>
<td>0.00064</td>
</tr>
<tr>
<td>S-7 clone 4</td>
<td>1</td>
<td>0.1</td>
<td>0.26</td>
</tr>
<tr>
<td>Recombinant B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-7 clone 1</td>
<td>0.3</td>
<td>7</td>
<td>0.22</td>
</tr>
<tr>
<td>S-7 clone 2</td>
<td>2.1</td>
<td>30</td>
<td>0.22</td>
</tr>
<tr>
<td>S-7 clone 3</td>
<td>0.16</td>
<td>3</td>
<td>0.015</td>
</tr>
<tr>
<td>S-7 clone 4</td>
<td>0.9</td>
<td>11</td>
<td>0.053</td>
</tr>
<tr>
<td>ts-2</td>
<td>0.1</td>
<td>0.15</td>
<td>0.001</td>
</tr>
<tr>
<td>ts-20</td>
<td>3</td>
<td>0.007</td>
<td>0.00018</td>
</tr>
<tr>
<td>ts+</td>
<td>4</td>
<td>0.007</td>
<td>1.04</td>
</tr>
</tbody>
</table>

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progeny. Thus, the ts-3 contents of the recombinant clones can be calculated from the e.o.p. to be <0.57%, in some cases <0.12%.

All of 24 ts+ clones from the ts-2 × 20 cross were as sensitive as ts+ to S-7 at concentrations up to 2.5 μg/ml. For example (Table 1), the e.o.p. (2.5:0 μg/ml) at 37 °C were 0.0063 (ts+ and ts-20), 0.0012 to 0.01 (recombinants; mean = 0.0027) and 0.11 (ts-2). Thus the ts-2 contents of the recombinant clones can be calculated to be <2.5%, in some cases <1.1%. Two of these recombinants (e.o.p. in 2.5 μg/ml S-7 of 0.003) were recloned at 37 °C in presence of S-7 (2.0 μg/ml), and four of these ‘S-7 isolates’ from each recombinant were compared with ts-2 by three tests (Table 2). The isolation procedure was interesting in that it selected from the ts+ recombinants a large number of mutants in these three characters, particularly S-7-dependent ts mutants, which are probably defective in structural protein, but no isolate had the combination of characters of ts-2, and so in these two recombinant clones the ts-2 content was <0.6%.

Nine overtly guanidine-sensitive clones were identified by spot testing the ts+ recombinant clones isolated from the cross ts-3 × 28g. When checked by full plaque assay, all were
Fig. 3. Sucrose-gradient sedimentation of virus RNA labelled with $[^{3}H]$-uridine from purified virus particles of a $ts^+$ recombinant clone isolated from the cross $ts^{-3} \times 20$. The top of the gradient is at the right: 17 h at 42 000 g and 25 °C in the Spinco SW 27 rotor, 15 to 30% sucrose gradient in NETS buffer (see Methods). The arrow shows the position of 28S ribosomal RNA.

as sensitive as $ts^+$ to guanidine carbonate (Table 1): the e.o.p. (150:10 µg/ml) at 37 °C were 0.0001 ($ts^+$ and $ts^{-3}$), 0.00001 to 0.0002 (recombinants) and 1.0 ($ts^{-28g}$). Thus the $ts^{-28g}$ contents of the guanidine-sensitive recombinant clones can be calculated to be < 0.01%, in some cases < 0.001%. Similar findings were obtained in a previous test with the cross $ts^{-149} \times 28g$ (Cooper, 1968); 42 unselected $ts^+$ recombinant clones tested were either as sensitive as $ts^+$ or as resistant as $ts^{-28g}$ to guanidine, with no intermediate sensitivities. No evidence for segregation behaviour was obtained from these guanidine tests.

Comparison of recombinant and revertant sensitivities to the oxadiazole derivative

Eleven $ts^+$ plaques were picked from a 39.5 °C assay of a $ts^{-3}$ self-cross, and incubated in tube cultures for 2 days at 37 °C. Titres were then 3 to 16 $\times 10^6$ p.f.u./ml, and the e.o.p. (39.5 °C:37 °C) all 1 to 2. The sensitivities of these revertants to the oxadiazole derivative were compared in concurrent assays with those of the $ts^+$ recombinants isolated from the cross $ts^{-3} \times 20$ and described above. Fig. 2 (left) shows that the distribution of the sensitivities to the oxadiazole derivative of the revertants was quite different from that of the recombinants, the revertants' sensitivities covering a much wider range. The revertants' e.o.p. (10:0 µg/ml) were spread from 0.005 to 0.1 (mean = 0.0265), while the recombinants' e.o.p. (10:0 µg/ml) ranged from 0.0012 to 0.0076 (mean = 0.0036); $ts^{-3}$ was 0.41, while $ts^+$ and $ts^{-20}$ were 0.01 and 0.0075, respectively. This difference in distribution of sensitivities indicates that the presumed $ts^+$ recombinants were unlikely to have arisen by an enhanced rate of reversion of $ts^{-3}$, supporting the presumption that they were in fact recombinants.
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Since all the recombinants isolated were oxadiazole-sensitive, the oxadiazole-resistance locus of ts-3 must be very close to or to the right of ts-3 in the genetic map, i.e. in structural protein (Cooper et al. 1971). The ts+ revertants from ts-3 were also all changed in sensitivity to the oxadiazole derivative (Fig. 2), a covariation confirming the location of its ts defect and oxadiazole resistance in the same gene, namely structural protein (see discussion of an analogous covariation of guanidine sensitivity with structural protein character by Cooper, Wentworth & McCahon, 1970b). Thus the oxadiazole derivative is yet another inhibitor whose action is implicated to depend on the configuration of a product of the structural protein gene, an effect with presumptive relevance to the proposed regulator of poliovirus (Cooper, Steiner-Pryor & Wright, 1973). The sensitivities of many of the revertants were intermediate between those of ts+ and ts-3, presumably reflecting the effects of internal suppression.

**Sedimentation coefficient of RNA from a ts+ recombinant**

Several of the ts+ recombinant clones isolated from the cross ts-3 × 20 and described above were grown in suspension cultures containing [3H]-uridine. The procedure is described in the Methods. The virus was purified, RNA liberated by SDS treatment and its size examined by its sedimentation behaviour in SDS-sucrose gradients (Fig. 3). The bulk of the label sedimented at about 35 S, in a pattern indistinguishable from that of ts+ (Cooper et al. 1970a).

**DISCUSSION**

In many instances in biology, the existence of genetic recombination can only be inferred from the observation of double mutants in the progeny of a cross in significant excess over spontaneous mutation. For poliovirus, the proportion of excess double mutants is high (up to 30 times the background), reproducible and characteristic of the parental pair, and the genetic map derived from it has many properties (mentioned below) that give confidence in this inference. A number of conceivable artefacts that might give the same result, e.g. the presence of clumps, complementation and/or recombination in the assay plates or some hypothetical mechanism for enhancement of reversion, can be ruled out by using appropriate procedures and reconstruction experiments (Cooper, 1968), and by the experiments of this paper. The unknown underlying mechanism is the subject of this discussion.

Fig. 4 illustrates eight simple ways in which a recombinant poliovirus particle might contain a full set of genetic information. The models include heterozygotes (all except e and f), unlinked reassortants (a, c, e, g) and covalently joined recombinants (b, d, f, h). Only stable, end-product particles representing the majority of the recombinants are taken into account, so that the possibility of linkage or elimination of genetic fragments during the mating events, before initial maturation or even during subsequent cloning and making of stocks, must be regarded as a part of the overall mechanism of recombination.

The data presented in this paper enable most of the models of Fig. 4 to be discarded. Models a and b, and simple aggregation of virus particles, are not permitted by the close physical resemblances of recombinant and parental particles shown by the data in Fig. 1. Model c is ruled out, and d made unlikely, by the lack of genetic segregation: we failed to find any (i.e. in some cases <0.1 %) parental phenotype among the progeny of 53 recombinant clones, although only one parent was selected for. Models e and g received no support from the sedimentation behaviour of recombinant RNA, as the majority situation.

Models f and h remain, both involving covalent linkage. The segregation tests suggest that h (and g also), if they occur at all, are rare, since none of 53 recombinant clones had the dominant (i.e. drug resistant) parental phenotype, and an undetectably small proportion
Fig. 4. Schematic illustrations of possible RNA composition of poliovirus recombinants. The thick and thin lines denote the genotype of each parent, and the length (e.g. of the thick line in c) is equivalent to the length of the genome of \(ts^+\). Models a to h represent eight of the simplest possible ways in which a full, non-defective set of poliovirus genetic information could be derived from defective parents and included in a single recombinant particle.

(in some cases <0·1%), of their progeny was drug resistant. Accordingly, either < 2% of the recombinants were heterozygous for the region selected by the inhibitor, or a larger proportion was heterozygous but the duplicated region comprised a very small proportion of the genome (in some cases < 0·1%, or < 8 nucleotides). At all events, gene duplication cannot be extensive in the large majority of the recombinants.

In all these tests, the recombinants were not distinguishable from conventional virus particles, leading to the conclusion that the RNA of the large majority of poliovirus recombinant particles appears to be in a single covalently joined piece of about the same size as the parental RNA. That viable picornavirus RNA is able to exist in fragments exhibiting genetic complementation is in any case made most unlikely by several aspects of the molecular biology of poliovirus to be found in the literature.

Perhaps the outstanding feature of poliovirus recombination is its apparently complete conventionality, in terms of, for example, many DNA systems. The genetic map is linear, additive and reproducible, and thoroughly self-consistent. Many data on a relatively large number of mutants (about 40) involving recombination, physiological and inhibitor studies (Cooper, 1969; Cooper et al. 1971; Summers & Maizel, 1971; Taber, Rekosh & Baltimore, 1971), all gave the same results for relation between map position, \(ts\) defect and 5′-3′ orientation. The unexpected locus for guanidine resistance in structural protein (Cooper, 1968) led to predictions for a regulator (the ‘equestron’), some of which have been verified by other means (Cooper et al. 1973; Steiner-Pryor & Cooper, 1973; Wright & Cooper, 1974). The additivity of recombination frequencies could not be obtained without equal and reciprocal involvement of both parental strands, although direct evidence for this is lacking. The low recombination frequencies (< 1% \(ts^+\) recombinants) are to be expected from the small genome (7500 nucleotides), and the frequencies per codon are in fact not very different from those of DNA phages.

These factors and the present results argue strongly in favour of a precisely ordered molecular exchange mechanism for genetic recombination of poliovirus, similar in end result to that of DNA systems (Hotchkiss, 1971). This may be effected by novel RNA-processing host enzymes analogous to those for DNA breakage–reunion, and such enzymes have
been inferred or reported to exist in bacterial and mammalian systems (Závadová, 1971; Béchet, 1972; Silber, Malathi & Hurwitz, 1972; Cranston, Malathi & Silber, 1973). Recourse to such enzymes may or may not be necessary, however. Because of the limited base-pairing in the poliovirus replication complex (Öberg & Philipson, 1971), we suggest the possibility that template dissociation in vivo could be appreciable. Thus poliovirus recombinants could well arise by reassortment of nascent chains plus replicase between templates, followed by precise realignment and continued replicase action on the new template. This is a form of copy choice. The question of the precise nature of the ordering mechanism for genetic recombination in poliovirus thus seems to be open for the present.

REFERENCES


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