Attempts to Extend the Genetic Map of Poliovirus Temperature-sensitive Mutants

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SUMMARY

Eighteen new ts mutants of poliovirus have been isolated after a variety of mutagenic treatments, and their loci identified in relation to the previous genetic map. The map was only extended by 25%, and the physiological characters of the new isolates corresponded in all aspects tested with those of the previous isolates. Apparently single mutants at the extreme left of the map were defective in synthesis of both double- and single-stranded RNA, functions that do not co-vary in other mutants. Two procedures respectively predicted to induce mutations preferentially in the 5' and 3' regions of the genome gave isolates which all indicated that the structural protein region was nearest the 5' end. The loci for resistance to dextran sulphate and to ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate both lie in the structural protein region.

INTRODUCTION

A genetic map has been obtained by recombination analysis of 19 ts mutants of poliovirus (Cooper, 1968). Their properties indicated that those in the right-hand half were defective in structural protein, while those in the left-hand half had defects in RNA synthesis (Wentworth, McMahon & Cooper, 1968; Cooper, 1969; Cooper, Stancek & Summers, 1970a; McMahon & Cooper, 1970; Cooper et al. 1971).

This map revealed a surprisingly small number of gene functions, giving rise to the suspicion that it might represent only a small portion of the poliovirus genome. Such a possibility was examined in two ways: (i) by using independent methods to estimate the scale of the genetic map (Tannock, Gibbs & Cooper, 1970; Cooper & Bennett, 1973), and (ii) by initiating a second mutant isolation programme, using different mutagenic procedures and making attempts to mutagenize the 3' and 5' regions preferentially in order to extend the boundaries of the map.

This paper describes the results of the second approach, involving the isolation and relation by recombination to the first genetic map of a further 18 ts mutants of poliovirus, mostly derived by different mutagenic procedures. In addition, we report the position in this map of two more characters: resistance to dextran sulphate (m+) and to a synthetic thiopyrimidine (S-7; Yamazi, Takahashi & Todome, 1970), together with some physiological characters of the new mutants.

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METHODS

**Virus growth and assay procedures** (using the human amnion U cell line). The standard recombination test and the preparation of virus stocks for this test were as described previously (Cooper, 1968). The cy character (low e.o.p. in 0.01 mM-cystine compared with 0.1 mM-cystine), and the thermolability (ΔH) character, using stocks in which stabilization by cystine was avoided, were determined as described by McCahon & Cooper (1970). The pti character (defect in prevention of host cell DNA synthesis) was determined in tube cultures as described by Cooper, Johnson & Garwes (1966), and the methods for analysis of virus RNA synthesis were described by Cooper *et al.* (1970a). Dextran sulphate had a mol. wt. of $2 \times 10^6$ ('2000', Pharmacia, Uppsala).

**Mutant isolation.** Plaques were picked from 37 °C assays of mutagenized ts+ (or in one case a guanidine-resistant derivative, ts+g) stocks, or of mutant-free RNA, and likely ts isolates were identified by spot tests on pre-poured agar cell suspension cultures (2 days at 37 °C or 39·5 °C in parallel assays). These were examined further by full plaque assay at 37 °C and 39·5 °C, and the confirmed ts mutants were recloned twice and recombination stocks prepared. The ts isolation rates given refer to all the confirmed ts mutants obtained, but only a small proportion of these from all mutagenic procedures was suitable for genetic mapping and accordingly for inclusion here. Criteria of suitability included a leak rate of $< 0·1\%$, a content of ts+ revertants that was low ($< 0·1\%$) but measurable, and apparent singularity of ts defect. The latter was judged by the formation of plaques of wild-type character in assays at 39·5 °C both by the ts+ revertants and by ts+ recombinants produced in crosses with known single ts mutants (see Cooper, 1968, for criteria). In a few cases, singularity was also testable by the approximate additivity of recombination frequencies. The mutants were isolated by a variety of mutagenic procedures, each repeated on several occasions, as follows.

**Continuous incorporation of 5-fluorouracil (5FU).** Some further mutants were examined from the series isolated by growth in 5FU as previously described (Cooper, 1964; Cooper *et al.* 1966): ts-24 (4 mm-5FU); ts-81, -123, -147, -151, -182 (1 mm-5FU).

**Hypothetical 3'-pulse of 5FU.** A procedure was followed that was expected to introduce mutations preferentially in the 3' region of virus RNA (Cooper *et al.* 1971). Cells were incubated at 2·5 $\times 10^6$/ml for 30 min at 37 °C in Eagle’s medium containing 5% calf serum, 0·7 mg/ml NaHCO3 and 10 mM-5FU, chilled, centrifuged and infected with ts+ (5 to 10 p.f.u./cell, 5 $\times 10^8$ cells/ml) at 0 °C in the same medium containing 100 μg/ml cycloheximide. After 1 h, the cells were diluted to 5 $\times 10^5$ cells/ml into the same medium (including 10 mm-5FU and 100 μg/ml cycloheximide) at 37 °C, and after a further 1 h were chilled, washed twice and resuspended in warm medium containing 10 mm-uridine but no 5FU or cycloheximide. The culture was frozen after a further 6 h. Plaque assay plates of this stock were incubated under temperature-shift conditions (16 h at 37 °C then 48 h at 39·5 °C), when small plaques were picked and their progeny spot-tested. The total ts isolation rate was almost 0·5% (8 mutants), and two mutants (ts-201, -202) were suitable for mapping.

**Hypothetical 5'-pulse of 5FU.** A procedure was followed that was expected to introduce mutations preferentially in the 5' region of virus RNA (Cooper *et al.* 1971). Cells (2 $\times 10^6$) were infected at 0 °C with ts+g (5 to 10 p.f.u./cell) and incubated at 37 °C for 2 h at 10⁶/ml; 10 mm-5FU was added and the cells were chilled 2 min later, disrupted in 5 ml vol. by Dounce homogenization and virus RNA species extracted from the cytoplasm with phenol in 0·01 M-EDTA, 0·14 M-NaCl, 0·01 M-phosphate, pH 7·3 (at 60 °C for 3 min). After cooling, the aqueous extract was treated with 10 μg/ml ribonuclease (30 min at 25 °C), extracted
twice more with phenol-EDTA plus 1 mg/ml bentonite (at 60 °C for 10 min), and the
RNA precipitated at 4 °C with 1/10 vol. 2 M-NaCl + 2 vol. ethanol. The precipitate was
dissolved in 0·5 ml 0·14 M-NaCl, 0·01 M-phosphate, pH 7·3, containing 100 μg/ml DEAE-
dextran. Five monolayer cultures of U cells (5 × 10⁶ cells) were washed twice with 0·14 M-
NaCl, 0·01 M-phosphate, pH 7·3, treated with the same buffer containing 100 μg/ml DEAE-dextran (1 ml, for 10 min at 37 °C), and infected with 0·1 ml of the RNA after removing
the bulk of the buffer. The medium was then replaced and the cultures re-incubated for
1 cycle of growth (for 6 h at 37 °C), from which progeny clones were isolated without any
selective criteria. The total ts isolation rate was about 15 % (32 mutants), and one mutant
(GT82) was suitable for mapping.

Nitrous acid. Nitrous acid mutants were obtained by mutagenesis of both intact virus
and free (infectious) RNA. A stock of ts + (2 × 10⁸ p.f.u./ml) at 18 °C was made 1 M in NaNO₂
and 0·125 M in Na-acetate (pH 4), and samples were diluted 1/50 in cold Eagle’s medium
containing 10 % calf serum at 1 min intervals for plaque assay. Inactivation was exponential
to 4 min (5 × 10⁻⁸ survivors), and clones were isolated from the 3 and 4 min samples for
spot test. The total ts isolation rate was about 3 % (7 mutants), and three mutants (ts-501,
-503, -505) were suitable for mapping.

Free RNA was prepared from a stock of ts + as described by Wentworth et al. (1968),
twice precipitated in ethanol and stored in 0·14 M-NaCl, 0·01 M-phosphate, pH 7·3, 0·01 M-
EDTA at −45 °C. Samples were treated with an equal vol. of HNO₂ (m-Na acetate, pH 4·5,
+4 M-NaNO₂, 1:1) at 20 °C, and the reaction stopped at intervals by diluting 1/100 in
0·14 M-NaCl, 0·01 M-phosphate, pH 7·3, 100 μg/ml DEAE-dextran at 0 °C. The inactivation
curve was biphasic, showing a rapid drop to 25 % survival by 30 s then a slow exponential
inactivation to 8 % survival by 600 s. Control RNA was unaffected. In one type of isolation
procedure, the surviving RNA sampled at intervals was assayed by the agar cell-suspension
method using DEAE-dextran (Koch, Quintrell & Bishop, 1967) in 88 mm Petri plates.
Small plaques were picked from samples taken after 2 and 3 min treatment (giving about
20 % survival) and assayed by temperature-shift incubation (16 h at 37 °C then 48 h at
39·5 °C). Their progeny was then spot-tested. In a second isolation procedure, the infec-
tious RNA surviving 5 min treatment was passaged once in monolayer culture and the
virus progeny were cloned. The total ts isolation rate was about 1 % in both procedures
(ten mutants), and three mutants (ts-523 from plating free mutagenized RNA, and ts-522
and -526 from plating the progeny of mutagenized RNA) were suitable for mapping.

Snake-venom phosphodiesterase. Infectious virus RNA was inactivated by very dilute
solutions of *Crotalus* phosphodiesterase in the hope of obtaining 3' deletion mutants. The
procedure and inactivation kinetics are described by Cooper et al. (1971); surviving RNA
was plated directly as above and mutants isolated from small plaques in 37 to 39·5 °C tem-
perature-shift assays as above. The total ts isolation rate was 10 %, 1 % and 2 % in three
experiments (7 mutants), and two mutants (ts-701, -702) were suitable for mapping.

*N-methyl-N'-nitro-N-nitrosoguanidine (NTG).* NTG was added to a ts + stock (2 × 10⁸ p.f.u./
ml) at 18 °C to 100 μg/ml, and samples were diluted 1/20 in cold PBS at intervals up to
210 min, when the titre had fallen to 8 × 10⁶ p.f.u./ml. The inactivation curve was first order
initially, the rate gradually slowing. The samples were dialysed, passaged once in monolayer
culture (to give about 10⁶ p.f.u./ml) and clones isolated for spot test. The total ts isolation
rate was about 0·5 % (5 mutants) from the highest sample dose (210 min), and one mutant
(NTG40) was suitable for mapping.

*S-7.* Ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate was a generous gift from
Toyama Kagaku Kogyo Co., Tokyo.

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RESULTS

Effect on the ts genetic map of different mutagenic treatments

The ts mutants whose isolation was described in Methods were prepared in the form of special recombination stocks of high titre and low revertant content, and analysed by the standard recombination test (Cooper, 1968). Fig. 1 shows a genetic map of those new mutants that were sufficiently stable and non-leaky, as well as having the required criteria of singularity, to be mapped in crosses with the main standard mutants of the previous study. Once again the key standard mutant was ts-28g. Those new mutants that could be mapped by assay in presence and absence of guanidine in crosses with ts-28g (circled) could be unambiguously assigned positions to the right or the left of ts-28 in the genetic map.
One new mutant (ts-8I) was located by analogous crosses with a newly developed guanidine-resistant variant of ts-20 (ts-20g). In several cases (ts-202, -182, -201, -8I, NTG40, GT82, ts-147) crosses with other standard mutants allowed the new mutants' positions to be confirmed by a reasonable degree of additivity of recombination frequencies, and these mutants are all expected to have a single ts lesion. Although the remainder of the new mutants gave no sign of a second ts lesion, this possibility has not been ruled out. Some new mutants (ts-24, -151, -123, -503) were too unstable or leaky to be mapped except in crosses with ts-28g followed by assay in presence of guanidine; such mutants can only be located if their defects lie between 28 and g. Six isolates (not described here) either failed to show any recombinants or gave non-additive recombination frequencies. They could not be used for mapping purposes and are presumed to contain multiple ts defects.

The new mutants were all located within the map obtained from the earlier series, with the three exceptions of ts-182, -201, and -202. The last two were the only products of the most extensive of the isolation series, in which nearly 2000 small-plaque isolates from temperature-shift plates were scanned in repeated trials for ts properties. This series represented an attempt to mutagenize specifically the 3' region of vRNA, but which was very poorly mutagenic. The procedure (see Methods) was designed to load the cell with 5FU while replication was suppressed by cycloheximide, then to release replication in the presence of a large excess of uridine. It was expected that a few molecules of polymerase would first be made, which would then catalyse RNA synthesis in a rapidly decreasing concentration of mutagen. Thus the first RNA to be polymerized (the 5' region of the complementary or cRNA strand) should incorporate the most 5FU, leading to preferential mutagenesis in the 3' region of the viral plus (vRNA) strand. The number of isolates obtained (two) was inadequate for statistical interpretation, but since both mutants were located far to the left of the genetic map they suggest that this is the 3' end. They have extended the size of the map by 25%.

Apart from this, the ts map has not been modified by use of different mutagenic treatments, and the mutants obtained with NTG, or with HNO3 treatment of virions or RNA, or with phosphodiesterase (probably spontaneous mutants, see below) did not differ from those obtained with 5FU.

Following the isolation of mutants possibly located in the 3' region of the genome, an attempt was made to mutagenize preferentially the 5' region. The procedure (see Methods) was designed to allow a brief pulse of incorporation of 5FU without any chase period, and then to extract the RNA and destroy all except double-stranded RNA (DS-RNA) by treatment with ribonuclease in relatively high salt concentrations. It was expected that the residual DS-RNA would comprise two species: one species would contain an intact vRNA or cRNA strand hybridized with RNA fragments (produced from the replicative intermediate; Öberg & Philipson, 1971) and the other would comprise the 'replicative form' (RF), in which both strands of the duplex were intact. Examination of the various possibilities shows that the only mutagenized intact strands will have 5FU incorporated in the 3' region, i.e. the parts of either vRNA or cRNA strands that were completed during a pulse of mutagen. Internally mutagenized RNA strands are expected to be incomplete and therefore non-infectious. It was assumed that intact cRNA strands do contribute substantially to the infectivity of DS-RNA, i.e. are transcribed at an early stage (see Discussion).

This procedure proved to be unusually mutagenic. Table I summarizes the properties of 75 mutants that were found among 200 random isolates tested; 32 of them were ts. At least 29/32 ts mutants were changed in structural protein (cy+ → cy), and a further 43 were also
Table I. Characters of 200 random clones from poliovirus strain ts+g cy+
undergoing hypothetical 5' mutagenization

<table>
<thead>
<tr>
<th>Strain characters</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts+g cy+ (parental)</td>
<td>125</td>
</tr>
<tr>
<td>ts g cy+</td>
<td>3</td>
</tr>
<tr>
<td>ts+g+cy+</td>
<td>34</td>
</tr>
<tr>
<td>ts+g cy</td>
<td>5</td>
</tr>
<tr>
<td>ts g cy</td>
<td>29</td>
</tr>
<tr>
<td>ts+g cy</td>
<td>4</td>
</tr>
<tr>
<td>ts g+cy</td>
<td>0</td>
</tr>
<tr>
<td>ts g+cy</td>
<td>0</td>
</tr>
</tbody>
</table>

identified as structural protein mutants (g → g+, cy+ → cy, or both). One strain (GT82,
ts g cy) was sufficiently stable and non-leaky to be mapped, and its ts defect was located
close to that of ts-3, i.e. in the structural protein region. Thus, within the limits of the
assumption made, the hypothetical 5' mutagenesis gave the same result as the hypothetical
3' mutagenesis, namely that the 3' region lies to the left of the genetic map.

The venom phosphodiesterase treatment apparently did not yield ts mutants with 3'
deletions, as the isolates showed a normal revertant content (10^{-2} to 10^{-5} ts+),
and the two mutants mapped were located in the centre of the map (Fig. 3). These isolates are presumed
to be spontaneous mutants. This work was done before the presence of 3' terminal poly
A sequences was known (Yogo & Wimmer, 1972), but it now appears that much of this
material is required for infectivity of poliovirus RNA (Spector & Baltimore, 1974). In our
work the enzyme was used in sufficiently high dilution to render endonuclease action
unlikely.

Physiological characterization of the new poliovirus ts mutants

The members of the first isolation series of poliovirus ts mutants showed a self-consistent
correlation between map position and many different physiological characters (Cooper,
1969). In order to confirm their position in the genetic map, certain physiological tests were
applied to the more significant of the new mutants. The results are summarized in Table 2,
and show that, without exception, mutants of the second series show the same correlation
as those (underlined) of the first series. As before, the cy character (dependence of plaque
formation on the presence of 0.1 mM-cystine; McCahon & Cooper, 1970), was restricted to
mutants in the right-hand (structural protein) region of the map, as was heat-lability
(ΔH character). As before (Cooper et al. 1966), the new mutants in the left-hand region
were also defective in prevention of host cell DNA synthesis (pti character). These mutants
were covariantly defective in c.p.e. (ib character; Garwes, Wright & Cooper, 1975).

Mutants ts-201 and -202 were examined for [3H]-uridine incorporation by the same
temperature shift-up procedure described by Cooper et al. (1970a). In this test, virus replication
was permitted in presence of 0.3 μg/ml actinomycin D until ample cRNA and vRNA tem-
plates were available (2 h at 36.4 °C), then half of the cultures were transferred to 39.2 °C,
label added (6 μCi/ml) 15 min after transfer and the RNA species made after a further 2 h
at permissive and restrictive temperatures examined by rate zonal sucrose gradient sedi-
mentation (Fig. 2). The results were identical to those for ts-20 (Cooper et al. 1970a), i.e. no
new RNA (single- or double-stranded) was made at restrictive temperature. This double
defect is of considerable interest, since the additive recombination frequencies with the new
mutants to the left (Fig. 1) show that both ts-20 and -201, at least, are single mutants. Fig. 2
shows a frequent finding, namely that the mutants were also somewhat defective at permis-
sive temperature and produced low yields of RNA and relatively more debris than the ts+
control. A reconsideration of the data on ts-81 reported by Cooper et al. (1970a) shows
that this mutant should be regarded as defective in production of DS-RNA, since although
No more room in the poliovirus ts map

Table 2. Summary of physiological characters of the new poliovirus ts mutants in comparison with standard mutants of the previous isolation series (underlined)*

<table>
<thead>
<tr>
<th>Character</th>
<th>ts+</th>
<th>202</th>
<th>201</th>
<th>20</th>
<th>81</th>
<th>28</th>
<th>151</th>
<th>123</th>
<th>GT82</th>
<th>2</th>
<th>104</th>
<th>149</th>
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<tbody>
<tr>
<td>cy†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ΔH†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>.</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pti†</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DS.RNA§</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>.</td>
<td>+</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SS.RNA§</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-7¶</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* '+' = wild type; '-' = mutant; '.' = not tested.
† Either from results in, or determined by methods from, McCahon & Cooper (1970).
‡ Either from results in, or determined by methods from, Cooper et al. (1966).
§ Either from results in, or determined by methods from, Cooper et al. (1970a).
¶ From Garwes et al. (1975).
†† From Table 3.

Fig. 2. Analysis of virus RNA species made by ts+ (a, b), ts-201 (c, d) and -202 (e, f) at permissive (36.4 °C, a, c, e) and restrictive (39.2 °C, b, d, f) temperatures. The tops of the gradients are to the right; arrows show the position of 28S and 16S ribosomal RNA as measured by extinction traces at 260 nm from a Gilford recording spectrophotometer. Infected cell cytoplasms labelled with [3H]-uridine were heated at 60 °C for 3 min in presence of 1% SDS, and portions derived from equivalent numbers of cells were centrifuged through 25 to 30% (w/w) sucrose gradients in 0.1 M-tris, pH 7.4, 0.01 M-EDTA and 5 mg/ml SDS for 16 h at 60000 g in a Spincro SW25.3 rotor at 23 °C.
considerable amounts of DS-RNA were made; it was mostly in fragments. This conclusion is recorded in Table 2.

Temperature shift-up experiments, assaying for infectivity by the same procedure described previously (Cooper et al. 1966), were also done with ts-201 and -202 (results not shown), revealing that these were ‘late mutants’ and the same as all but one of the first series. As before, one can only conclude that the function in which their defect lies still helps to make new progeny at a time when maturation has already begun.

The locus for resistance to dextran sulphate (m+ character)

High mol. wt. dextran sulphate prevents growth of some but not all poliovirus clones (Takemoto & Liebhaber, 1962). Virus particles of sensitive strains bind dextran or Sephadex sulphates more strongly than resistant strains (Bengtsson & Philipson, 1963; Bengtsson et al. 1964; Bengtsson, 1966), and the sensitive step in the growth cycle is the first (reversible) adsorption to cells and not subsequent irreversible attachment or RNA replication (Bengtsson, 1965). The dextran-sensitive (m) locus therefore occurs in genes that specify structural protein. In three-factor crosses, Bengtsson (1968) has shown the presence of more than one m+ locus, lying astride the locus for resistance to horse-serum inhibitor (ho), which is also in structural protein (Takemoto & Habel, 1959; Pagano, 1965).

The poliovirus strains described above (ts+ and ts derivatives) are all dextran sulphate-sensitive (m). In order to locate the m+ locus in the genetic map, attempts were made to select resistant (m+) clones from ts strains by picking plaques that appeared (usually with e.o.p. of 10^-4) in various concentrations of dextran sulphate. Resistant strains were readily obtained, and probably represent single mutations. However, all m+ strains from ts structural protein mutants were found to have reverted to ts+, and we were unable to obtain such tsm+ strains. This covariation is evidence that, as expected, these mutations lie in the same gene, and probably reflect intragenic suppression resulting from alterations in configuration of structural protein. The same restriction had previously been found in the isolation of g derivatives (B. B. Wentworth & P. D. Cooper, unpublished data); covariation between the g character and structural protein is discussed elsewhere (Cooper, Wentworth & McCahon, 1970c).

Two suitable m+ strains (ts-28m+; ts+m+) were obtained; ts-28m+ was recloned twice, recombination stocks with low ts+ content prepared and analysed by the standard recombination test. The plating efficiency of ts-28m+ at 37 °C was unaffected by dextran sulphate (250 µg/ml); it had the same plating characteristics (leak and wild-type content) at 39·2 to 39·5 °C as ts-28 and failed to yield any ts+ progeny in excess of spontaneous reversion in crosses with ts-28g. Presumably therefore its ts defect had remained unchanged during the selection procedure. However, ts+m+ progeny were obtained in 20-fold excess of spontaneous reversion in crosses with ts-3 and ts-149, when the progeny of these crosses were plated at 39·5 °C in presence of dextran sulphate. The recombination frequencies were always slightly in excess of those for ts'g recombinants obtained in concurrent crosses of ts-28g×ts-3 and ts-28g×ts-149, which were used as described by Cooper (1968) to standardize the ts+m+ frequencies. The mean standardized ts+m+ frequencies are recorded in Fig. 1. The ts+m+ recombinants turned out to be dextran sulphate-dependent at 39·5 °C, and so the total ts+ recombination frequency in crosses involving ts-28m+ could not be measured at 39·5 °C in the absence of dextran sulphate.

The close linkage of these particular m+ and g loci was confirmed by six replicate crosses between ts-28g and ts-28m+. These tests yielded gm+ double mutants in about 20-fold excess over the self crosses when assayed at 37 °C in presence of both guanidine and dextran.
No more room in the poliovirus ts map

Table 3. Sensitivity of single poliovirus ts mutants to S-7 that was incorporated in the agar of plaque assays

<table>
<thead>
<tr>
<th>ts</th>
<th>Defect</th>
<th>e.o.p. (10 μg/ml S-7:0)</th>
<th>S-7 character</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td></td>
<td>0.002</td>
<td>+</td>
</tr>
<tr>
<td>202</td>
<td>RNA replication</td>
<td>0.0006</td>
<td>+</td>
</tr>
<tr>
<td>182</td>
<td>RNA replication</td>
<td>&lt; 0.01</td>
<td>+</td>
</tr>
<tr>
<td>201</td>
<td>RNA replication</td>
<td>0.0007</td>
<td>+</td>
</tr>
<tr>
<td>523</td>
<td>RNA replication</td>
<td>0.019</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>RNA replication</td>
<td>0.0002</td>
<td>+</td>
</tr>
<tr>
<td>702</td>
<td>RNA replication</td>
<td>0.01</td>
<td>+</td>
</tr>
<tr>
<td>81</td>
<td>RNA replication</td>
<td>0.003</td>
<td>+</td>
</tr>
<tr>
<td>99</td>
<td>RNA replication</td>
<td>0.0001</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>RNA replication</td>
<td>&lt; 0.01</td>
<td>+</td>
</tr>
<tr>
<td>46</td>
<td>RNA replication</td>
<td>0.012</td>
<td>+</td>
</tr>
<tr>
<td>96</td>
<td>RNA replication</td>
<td>0.009</td>
<td>+</td>
</tr>
<tr>
<td>44</td>
<td>RNA replication</td>
<td>0.118</td>
<td>+</td>
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<tr>
<td>150</td>
<td>RNA replication</td>
<td>0.026</td>
<td>+</td>
</tr>
<tr>
<td>701</td>
<td>RNA replication</td>
<td>0.017</td>
<td>+</td>
</tr>
<tr>
<td>94</td>
<td>Structural protein region</td>
<td>0.78</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Structural protein region</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>89</td>
<td>Structural protein region</td>
<td>0.0014</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Structural protein region</td>
<td>0.0002</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Structural protein region</td>
<td>0.0015</td>
<td>+</td>
</tr>
<tr>
<td>104</td>
<td>Structural protein region</td>
<td>&lt; 0.01</td>
<td>+</td>
</tr>
</tbody>
</table>

The frequency was 0.180% after correction for spontaneous reversion. Concurrent crosses used as standards were six replicates of ts-28g × ts+, and these yielded 0.696% ts+g recombinants after background correction; the standard frequency for ts+g recombinants from this cross is 0.364% (Cooper, 1968), and normalization in terms of this gave a standardized gmr+ frequency of 0.094% (Fig. 1).

These crosses indicate a mean map position near g for this m+ locus, probably somewhat to the right, and falling as expected in the structural protein region of the ts genetic map.

The locus for resistance to ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate (S-7)

The synthetic thiopyrimidine S-7 has been shown to be a highly potent inhibitor of poliovirus, and resistant variants have been obtained (Yamazi et al. 1970). It therefore seemed likely to provide a good genetic marker, and resistant variants were sought from ts strains. However, tests of sensitivity revealed that three ts mutants (ts-2, -22, -94) were already resistant to S-7 (Table 3). The resistant mutants were restricted to those defective in structural protein. The relative resistance of ts+, ts-2 and -94 is compared more fully in Fig. 3; the absolute e.o.p. (10 μg/ml: 0) of ts+ varied between 0.001 and 0.015 in different tests. Fig. 3 also shows that a ts+ revertant from ts-94 (R+94) was altered in sensitivity towards that of ts+. This was the only ts+ revertant tested, but its covariation suggests that the ts and S-7 resistance loci of ts-94 were in the same gene, namely structural protein.

Mapping experiments with the S-7 locus of ts-2 have been described elsewhere (Cooper et al. 1974). These experiments showed that 24/24 ts+ recombinants isolated at random from a 39.5 °C assay (no inhibitors) of the cross ts-2 × 20 were as sensitive as ts+ to S-7, indicating that the S-7 resistance locus of ts-2 was close to, or to the right of, its ts locus in the genetic map, i.e. in structural protein. It was also reported that S-7 resistant clones selected from
Fig. 3. Sensitivity of poliovirus strains to various concentrations of ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate (S-7), in the plaque assay medium. Results are expressed as e.o.p. (plaques formed in presence: plaques formed in absence of inhibitor). ▲—▲, ts+; ○—○, ts-94; □—□, R+/94, a ts+ revertant from ts-94.

$ts^+$ strains were commonly changed in structural protein as well ($cy^+ \rightarrow cy$). In this connection, it may be mentioned that S-7 (10 g/ml) almost completely relieved the $ts$ defect of $ts$-3 in assays at 39.5 °C (results not shown). This property is shared with 0.1 mM-cystine, and strongly suggests that S-7 interacts with the defective gene product of $ts$-3, i.e. structural protein.

DISCUSSION

The result of the second mutant isolation programme, summarized by Cooper et al. (1971) and described in detail above, is virtually identical to that of the first genetic map. Thus increasing the number of mutants in the map about twofold, using different isolation procedures, has extended its boundaries only by 25%. This finding, and that of independent estimates of the scale of the genetic map (Tannock et al. 1970; Cooper & Bennett, 1973), together provide strong evidence that the genetic map does indeed represent the major part of the genome.

The results of attempts to mutagenize specifically the 3' or the 5' regions of vRNA are not amenable to statistical interpretation, and in any case depend upon several assumptions. Nevertheless, they are consistent in that all isolates indicate the coat protein region as being located nearest the 5' end. Since this work was completed, reports of studies with pactamycin inhibition of poliovirus translation (Summers & Maizel, 1971; Taber, Rekosh & Baltimore, 1971) have given the same result. Accordingly, these mutagenic procedures merit molecular investigation and possibly wider application.

More recently, Perez-Bercoff et al. (1974) have shown that mengovirus and infectious
mengovirus RF initiate their replication cycles in different ways. The results appear to rule out the possibility that the duplex strands of infectious RF may separate spontaneously to allow the vRNA chain to bind to ribosomes. Instead, they support the interesting alternative that a cellular polymerase may first transcribe RF as template. Since a cRNA transcript would not have messenger function (and is non-infectious, Roy & Bishop, 1970; Béchet, 1972a) it is to be expected that the transcript that actually establishes the infection initiated by RF is vRNA, and its template accordingly the cRNA moiety of RF. A similar conclusion is suggested by the finding that an intact cRNA chain of encephalomyocarditis virus when hybridized to non-infectious vRNA fragments becomes infectious (Béchet, 1972b). This therefore provides theoretical support for the apparent finding of preferential 5’ mutagenesis by the procedure given above, which is expected to introduce 5FU preferentially into the 3’ region of cRNA in RF. Examination of the progeny from an artificial heteroduplex RF derived from guanidine sensitive and dependent strains (Best, Evans & Bishop, 1972) suggested in contrast that the artificial duplex was unstable in vivo, as it had the phenotype of the vRNA strand. Presumably its bonding differs from the natural RF, and might perhaps be tested by sensitivity of its infectivity to actinomycin and ribonuclease.

Inhibitors of protein synthesis, such as p-fluorophenylalanine or puromycin (Scharff, Summers & Levintow, 1965) or cycloheximide (Ehrenfeld, Maizel & Summers, 1970), rapidly stop poliovirus RNA synthesis, showing that virus protein and RNA synthesis are strongly coupled in some way. The interpretation of this was uncertain, however. Our results with, for example, ts-20 show the reverse effect, namely that virus RNA synthesis is rapidly stopped after temperature shift-up, while virus protein synthesis continues (Cooper, Summers & Maizel, 1970b). Use of the more specific lesion of the RNA- mutants therefore uncouples RNA and protein synthesis in this system. Such a result suggests that the effect of the chemical inhibitors simply reflects a rapid turnover of virus replication enzymes or templates, rather than some more complex interaction between RNA and protein synthetic machinery.

The location of the dextran sulphate resistance character in the structural protein region of the genetic map is to be expected, and serves merely to consolidate previous findings. The new results with S-7 make it clear that sensitivity to the presence of this drug, like sensitivity to the presence of guanidine (Cooper et al. 1970c) and of 2-(3-chloro-p-tolyl)-5-ethyl-1,3,4-oxadiazole (Cooper et al. 1974) and to the absence of cystine (McCaohon & Cooper, 1970), is dependent upon the configuration of a product of the structural protein gene.

REFERENCES


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