THE CIRCULAR GENETIC MAP OF PHAGE S13

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Because of its small size[1] phage S13 is suitable for a study of all its genes and their functions. With this aim, conditionally lethal mutants of the suppressible (*su*) and temperature-sensitive (*t*) type have been isolated and classified into seven complementation groups, and the general function of five of the genes implicitly defined by these complementation groups has been determined.[8-10] This paper is concerned with the mapping of the seven known phage genes, with the ultimate aim of understanding both the organization of the phage genome and the mechanism by which it undergoes genetic recombination.

Although the DNA molecule of S13, as shown for the closely related phage ϕX174, is physically circular both in the single-stranded form of the mature virus[11] and in the double-stranded replicative form,[12, 13] this does not necessarily imply that the genetic map should also be circular since circular DNA is neither necessary nor sufficient for genetic circularity. On the one hand, a linear DNA molecule can give rise to a circular genetic map if the nucleotide sequences are circularly permuted;[14] an example is the T2–T4 phage system.[15-17] On the other hand, a circular DNA can yield a linear map if recombination involves opening of the ring at a unique site, as seems to occur for phage λ, which forms a closed DNA molecule after infection[18] yet has a linear vegetative and prophage genetic map.[19] In this report it will be shown that the genetic map of S13, as determined entirely by 3-factor crosses, is indeed circular, and, as might be expected for a circular genome, the occurrence of double recombination events appears to be the rule.

Materials and Methods.—Bacterial strains: *E. coli* C, a nonpermissive host for S13 *su* mutants;[20] *E. coli* C600.1, a permissive host for S13 amber mutants;[10] *E. coli* AB1157.6, an S13-sensitive derivative of AB1157 that suppresses most S13 amber mutants (AB1157 is a *rec*$^+$ strain[22] obtained from Dr. Paul Howard-Flanders and was made sensitive to S13 by a method previously described[23]); *E. coli* CA165.2, a permissive host for S13 ochre mutants;[24] *Shigella dysenteriae* Y6R, a permissive host for S13 amber mutants.[8]

Phage strains: All mutants employed in the mapping experiments have been previously described[8] with the exception of *su*N14, *su*N32, *ts*106.12, and *ts*205.26.1 (hereafter referred to as *su*14, *su*32, *t*'106 and *t*'205, respectively). *su*14 and *su*32 are nitrous acid-induced amber mutants in complementation group V. *t*'106 and *t*'205 are spontaneously occurring temperature-sensitive false revertants of *su*N106 and *su*HT205, respectively. *su*N106 is a nitrous acid-induced ochre mutant in group II, and *su*HT205 is a hydroxylamine-induced ochre mutant in group VI.[24] Neither *su*N106 nor *su*HT205 complements or recombines with its temperature-sensitive derivative. All *su* mutants used in the crosses were of the amber type (suppressible by C600.1 and Y6R).

Media: All media have been previously described.[8]

Isolation of double mutants: Only double mutants of the form *t su* were constructed. By ultraviolet irradiation of the parental phage to a survival of 2–4%, recombination between *t* and *su* mutants was stimulated 10- to 50-fold depending upon the particular combination of mutants used. Irradiated lysates were used to infect AB1157.6 growing exponentially (approximately 2×10^8/ml) in tryptone broth (TB) at a multiplicity of infection (moi) of 5 each. Adsorption was carried out at 37° in TB made 0.01 *M* in $CaCl_2$. After 10 min, the infected cells were diluted 100-fold into TB and incubated an additional 90 min at 37°. The bursts were then sterilized with $CHCl_3$ and assayed for wild-type recombinants and total phage. The frequency of wild-type recombinants var-

ied from 2×10^{-3} to 5×10^{-2}. The frequency of the double mutant in most of these crosses was found to be close to that of the wild type.

To facilitate isolation of the double mutant, the bursts were plated under conditions where both the *t* and *su* parents yielded plaques smaller than wild type. This was done by preadsorbing the phage to C600.1 and plating at temperatures between 37° and 40° on an indicator consisting of a 1:1 mixture of C600.1 and C overnight cultures. Under these conditions the *t* parent makes small plaques due to the elevated temperature, the *su* parent makes small plaques due to the presence of the nonpermissive cells, and, as expected, the double mutants make plaques that are on the average smaller than either parental type. Accordingly, by screening only small plaques there was an enrichment for the double mutants, which were recognized by their inability to make plaques on C at 37° or C600.1 at 43°.

The authenticity of presumptive double mutants was verified by backcrossing to each of the parents to show that no su^+ or t^+ recombinants were produced above the mutational background, which was $<10^{-4}$ % in all cases. Each presumptive double mutant was also checked to see that su^+ and t^+ revertants occurred in the stock at approximately the same frequencies as the parental stocks to preclude the possibility that the double mutants were really triple mutants carrying accidentally introduced *su* or *t* mutations that could affect the results of subsequent crosses.

Theoretical and procedural aspects of ordering triads by means of 3-factor crosses: The genes of S13 were mapped exclusively by 3-factor crosses of either the type (A) $su_at_1 \times t_2$ or (B) $t_asu_1 \times su_2$ so that the gene order could be deduced from qualitative results. Considering (A), there are three possible orders for the genetic markers: (1) su_a-t_1-t_2, (2) t_1-t_2-su_a, or (3) t_1-su_a-t_2. By choosing t_1 and t_2 in the same gene and su_a in another gene, order (3) can be eliminated and su_a can be referred to as the *outside marker;* the assumption involved is that two mutations in the same complementation group define a contiguous region of the genetic map uninterrupted by mutations in other groups. The choice between orders (1) and (2) is then determined by the relative frequencies of the su_a and su_a^+ alleles among $t_1^+t_2^+$ recombinants in either of the two crosses, (A1) or (A2), shown in Figure 1. The ratio, majority allele/minority allele of the outside marker, will be said to define a *segregation ratio.* In cross (A1), if a majority of the $t_1^+t_2^+$ recombinants are su_a^+, then the order of the markers is (1); if a majority are su_a, the order is (2). In cross (A2), in which su_a is combined with t_2 (reciprocal combination), there should be a qualitatively reciprocal result for the outside marker, thus providing a check on the consistency of the results. Analogous reasoning applies to the ordering of triads in crosses of type (B), in which the roles of *su* and *t* mutations are reversed from those in crosses of type (A).

The procedure for carrying out the 3-factor crosses was as follows: AB1157.6 was grown to approximately 2×10^8/ml in TB (in cases where the group IV mutants *su*105 or *su*113 served as the outside marker in a 3-factor cross, Y6R was used). The culture was made 0.01 *M* in $CaCl_2$ and infected with the two parents at an moi of 5 each. Adsorption was allowed to proceed for 10 min without aeration, and then the infected cells were diluted 100-fold into TB and 60 min later sterilized with $CHCl_3$. Adsorption and growth of the phage occurred at 37°. t^+ recombinants were then assayed on C600.1 at 43° or su^+ recombinants were assayed on C at 37°. t^+ recombinant plaques were sampled at random and then scored for their *su* allele by testing their ability to grow on C600.1 and C at 37°; su^+ recombinant plaques were also sampled at random and then scored for their *t* allele by testing growth on C at 37° and 43°.

In all 3-factor crosses, the frequency of wild-type revertants, as determined from self-crosses of the parental types, was less than 5% of the frequency of recombinants.

The relative output of the two parental types was not determined in the crosses since it was found that the segregation ratio of the outside marker was independent of moi's of the parental phage over a wide range of relative moi's (10:1 to 1:10). This was expected because the low recombination frequencies found in S13 (see below) imply that each recombinant phage is usually derived from a single pairwise mating

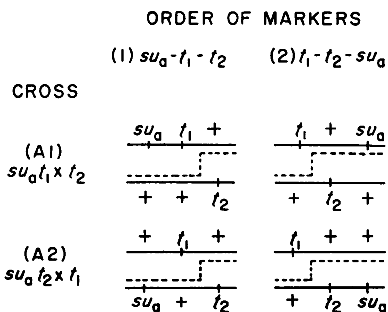

FIG. 1.—Distribution of outside marker in 3-factor crosses. Parental genome ———; recombinant genome - - - - - -.

event, so that in effect most recombinants arise from a subpopulation in which the two parental types are approximately equal in frequency.

Statistical analysis of segregation ratios: The actual numbers comprising the segregation ratios were tested against a 1:1 hypothesis by means of the χ^2 goodness of fit test. With the exception of crosses 17 and 18 in Table 1, the 1:1 hypothesis could be rejected at the 1% significance level.

Results.—The data from the 3-factor crosses are listed in Tables 1, 2, and 3. It can be seen in Table 1 that the mutant *su*14 is located at both ends of the composite genetic map; this finding is inconsistent with a linearly ordered map and leads to the conclusion that the genetic map of the phage is circular.

Additional evidence that linear ordering fails to hold for S13 markers and that the genetic map is circular is shown by the discrepancies between the order of triads predicted from a linear map and the results of direct crosses which are listed in Table 4. The fact that a gene can map both to the right and to the left of another gene suggests that both genes are located on a circular map. Similar arguments have been used to demonstrate circularity of the genetic map of phage T4.[14]

It should be noted that the ordering of every triad is based, at least or in part, on a segregation ratio favoring a mutant allele. Since the wild-type allele might predominate because of a selective advantage over the mutant allele in post-recombination events (e.g., some amber mutants are less efficiently suppressed at 43°), ordering a triad only on the basis of a segregation ratio favoring the wild-type allele of the outside marker would not be valid. Three cases have in fact been found (unpublished data) in which both reciprocal combinations of the outside marker result in segregation ratios favoring the wild type. On the other hand, only rarely would one expect a conditionally lethal mutant to enjoy a selective advantage over the wild type. In fact, in six distinct cases where the mutant allele predominated in one combination of the outside marker, the reciprocal combination was also tested, and it always gave a predominance of the wild-type allele.

A seventh gene (complementation group VI) of S13 has recently been discovered.[24] This gene is defined by a single ochre mutant, *su*HT205. Since the ochre-suppressing strain of *E. coli*, CA165.2, will not grow at 43°, it was not possible to use the ochre mutant as an outside marker in 3-factor crosses. Therefore, a spontaneously occurring temperature-sensitive false revertant of *su*HT205 designated *t'*205 was isolated, as described in *Methods*.

The mutant *t'*205 is unusual in that it is temperature-sensitive on C but not on C600.1. Because of this, it was possible to use *t'*205 as an outside marker in combination with either a pair of *t* or a pair of *su* markers, since it behaves like a temperature-sensitive mutant on C and like an amber mutant at 43°.

Isolation of double mutants containing the *t'*205 mutation was accomplished in the same manner as described in *Methods* except that the double mutant was recovered by screening for recombinants incapable of yielding wild-type revertants at a frequency greater than 5×10^{-9}, the reasoning being that since the frequency of revertants in the parental stocks was between 10^{-6} and 10^{-7}, the frequency of revertants in the case of the double mutant should be less than 10^{-12}.

The 3-factor crosses serving to locate the position of gene VI are tabulated in Table 2. The results indicate that gene VI is between gene I and gene V. A

TABLE 1

CROSSES DEMONSTRATING CIRCULARITY OF THE S13 GENETIC MAP

	Three-factor cross*	Selected progeny†	Number of Selected Progeny Carrying Each Allele of Outside Marker†: Wild-type allele	Mutant allele	Order Deduced
1.	*su*14(V)-*t*173(I) × *su*32(V)	*su*⁺	17	49	14-32- -173
2.	*su*14(V)-*t*173(I) × *t*526(I)	*t*⁺	7	41	14- -526-173
3.	*su*71(IIIa)-*t*526(I) × *t*173(I)	*t*⁺	27	115	526-173-71
4.	*su*43(IIIa)-*t*526(I) × *su*71(IIIa)	*su*⁺	125	268	526- -71-43
5.	*su*71(IIIa)-*t*526(I) × *su*43(IIIa)	*su*⁺	143	33	526- -71-43
6.	*su*71(IIIa)-*t*266(IIIb) × *su*43(IIIa)	*su*⁺	63	205	71-43-266
7.	*su*71(IIIa)-*t*330(IIIb) × *t*266(IIIb)	*t*⁺	8	40	71- -266-330
8.	*su*71(IIIa)-*t*266(IIIb) × *t*330(IIIb)	*t*⁺	73	23	71- -266-330
9.	*su*105(IV)-*t*266(IIIb) × *t*330(IIIb)	*t*⁺	5	71	266-330-105
10.	*su*113(IV)-*t*266(IIIb) × *t*330(IIIb)	*t*⁺	42	82	266-330- -113
11.	*su*113(IV)-*t*266(IIIb) × *su*105(IV)	*su*⁺	117	197	266- -105-113
12.	*su*105(IV)-*t*76(II) × *su*113(IV)	*su*⁺	8	54	105-113- -76
13.	*su*105(IV)-*t*76(II) × *t*′106(II)	*t*⁺	24	66	105- -106-76
14.	*su*113(IV)-*t*′106(II) × *t*76(II)	*t*⁺	43	5	113-106-76
15.	*su*113(IV)-*t*76(II) × *t*′106(II)	*t*⁺	9	38	113-106-76
16.	*su*14(V)-*t*′106(II) × *t*76(II)	*t*⁺	4	24	106-76-14
17.	*su*14(V)-*t*76(II) × *su*32(V)	*su*⁺	95	92	Indeterminate
18.	*su*32(V)-*t*76(II) × *su*14(V)	*su*⁺	98	91	Indeterminate

Composite Order:
(V)-(I)-(IIIa)- (IIIb)-(IV)-(II)-(V)
14-32-526-173-71-43-266-330-105-113-106-76-14

* Roman numerals in parentheses refer to the complementation group assignments of the mutants.
† In each cross, two of the markers are in one complementation group, the third being the outside marker. The selected progeny are always chosen as being recombinant for the two markers that are in the same group.

TABLE 2

MAPPING OF THE GROUP VI MUTANT *t*′205

	Three-factor cross	Selected progeny	Number of Selected Progeny Carrying Each Allele of Outside Marker: Wild-type allele	Mutant allele	Order deduced
1.	*su*14(V)-*t*′205(VI) × *su*32(V)	*su*⁺	29	101	14- 32-205
2.	*su*32(V)-*t*′205(VI) × *su*14(V)	*su*⁺	118	46	14- 32-205
3.	*t*173(I)-*t*′205(VI) × *t*526(I)	*t*⁺*	15	81	205-526-173

* *t*⁺ progeny were selected for on C600.1 at 43°, a permissive condition for the outside marker *t*′205.

TABLE 3

CROSSES VERIFYING THE CIRCULAR MAP

	Three-factor cross		Selected progeny	Number of Selected Progeny Carrying Each Allele of Outside Marker: Wild-type allele	Mutant allele	Ordered deduced
1.	*su*105(IV)-*t*526(I)	× *t*173(I)	t^+	92	56	105-526-173
2.	*su*105(IV)-*t*173(I)	× *t*526(I)	t^+	188	255	105-526-173
3.	*su*113(IV)-*t*173(I)	× *t*526(I)	t^+	42	114	113-526-173
4.	*su*86(II)-*t*173(I)	× *t*526(I)	t^+	31	76	86-526-173
5.	*su*86(II)-*t*526(I)	× *t*173(I)	t^+	80	16	86-526-173
6.	*su*105(IV)-*t*173(I)	× *su*113(IV)	su^+	54	86	105-113-173
7.	*su*105(IV)-*t*526(I)	× *su*113(IV)	su^+	52	144	105-113-526
8.	*t'*106(II)-*t'*205(VI)	× *t*76(II)	t^{+*}	15	81	106- 76-205
9.	*su*105(IV)-*t'*205(VI)	× *su*113(IV)	su^+	55	210	105-113-205

* + progeny selected for on C600.1 at 43°, a permissive condition for the outside marker *t'*205.

TABLE 4

ADDITIONAL EVIDENCE FOR A CIRCULAR MAP

Order predicted from the linear composite order in Table 1	Order from Direct Crosses: Table 3 crosses	Order deduced
*t*526-*t*173-(IV)	1, 2, 3	(IV)-*t*526-*t*173
*t*526-*t*173-(II)	4, 5	(II)-*t*526-*t*173
(I)-*su*105-*su*113	6, 7	*su*105-*su*113-(I)

diagram indicating the position of the seven known genes of S13 on the genetic map of the phage is shown in Figure 2. Polarity mutants in IIIa indicate that the genome is translated in a clockwise direction.[25]

Four types of checks for self-consistency of the map were performed. (In all that follow, when a pair of mutants is hyphenated it means that the left-right order is arbitrarily chosen as written.)

(1) Reciprocal combinations of the 4utside marker give reciprocal results. There are three pairs of such reciprocal combinations in Table 1 (crosses 4 and 5, 7 and 8, 14 and 15), one in Table 2 (crosses 1 and 2), and two in Table 3 (crosses 1 and 2, 4 and 5). In the case of each pair, one cross results in a majority of the selected progeny carrying the wild-type allele of the outside marker, whereas the reciprocal combination yields a qualitatively reciprocal result, i.e., a majority of the selected progeny carry the mutant allele of the outside marker.

(2) Different mutations in the same gene map on the same side of adjacent genes.

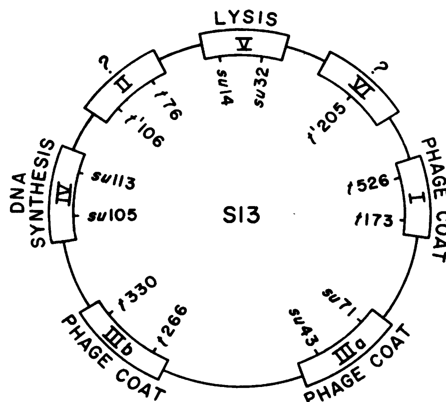

FIG. 2.—The genetic map of S13. The spacings between the genes have been drawn unequal in order to preserve the correct linkage relationships between genes I and IV (*su*105 must be closer to *t*526 than to *t*173 as implied by the results of crosses 1 and 2 in Table 3). The lengths of the boxes representing the genes are not proportional to actual map distances.

Recent tests of the heat stability of gene IIIa mutants indicate that IIIa, like I and IIIb,[10] also controls the structure of the phage coat.

There are two indications of this in Table 1 (crosses 9 and 10, 13 and 14). The group IV mutants *su*105 and *su*113 both map to the right of *t*266-*t*330 and to the left of *t'*106-*t*76.

(3) Crosses in which the outside marker is separated from the other two markers by one or more genes give orders consistent with the map. Four examples of this can be seen in Table 5, where the deduced orders on the left side of the table in conjunction with the map in Figure 2 allowed for the prediction of the orders given on the right side of the table. Since there are two possible results for any cross, the probability that the results of the four crosses were correctly predicted by chance alone is $^1/_{16}$. This low probability suggests that the predictions were truly verified and that the order of markers on the genetic map of S13 determined by 3-factor crosses, which was the basis for the predictions, is valid.

(4) Numerical values of segregation ratios are qualitatively in accord with the distances between selected and outside markers. Five different mutations have been employed as outside markers in combination with the group I mutants *t*526 and *t*173, and they each map to the left of *t*526-*t*173. These mutations listed in order of increasing distance from *t*526 are *t'*205, *su*14, *su*86, *su*113, and *su*105 (see Fig. 2). In combination with *t*526 and *t*173, they exhibited segregation ratios of 5.4 (81/15), 5.9 (41/7), 2.5 (76/31), 2.7 (114/42), and 1.4 (255/188), respectively. It can be seen that, within statistical error, the greater the distance between *t*526 and the outside marker, the lower the segregation ratio (i.e., the freer the assortment of the outside marker). Various mutations in combination with the group IV mutants *su*105 and *su*113 show the same effect. Listed in order of increasing distance from *su*113, the mutations are *t*76, *t'*205, *t*526, and *t*173. The corresponding segregation ratios are 6.8 (54/8), 3.8 (210/55), 2.8 (144/52), and 1.6 (86/54), respectively.

The indeterminate results of crosses 17 and 18 in Table 1 deserve special comment. The map in Figure 2 predicts that *t*76 should be closely linked to the left of *su*14-*su*32. The results of the indeterminate crosses indicate, however, that *t*76 assorts independently of *su*14, i.e., recombination in gene V is accompanied approximately 50 per cent of the time by recombination between gene II and gene V. But, as shown by the result of cross 16 in Table 1, genes II and V are linked. These results imply that the amount of recombination is not solely a function of the distance between markers, but is also dependent upon the particular region of the genome in which recombination is selected.

In view of the fact that the highest recombination frequency observed by E. S. Tessman in 2-factor crosses was less than 0.2 per cent,[8] the finding that almost all of the segregation ratios observed in 3-factor crosses are lower than 10:1 demonstrates a high positive correlation of S13 recombination events. This is further

TABLE 5

CONSISTENCY OF THE CROSSES IN TABLE 3 WITH THE GENE ORDER SHOWN IN FIGURE 2

Table 3 crosses	Order deduced	Prediction from Figure 2	Table 3 verifying crosses
1, 2, 3	(IV)-*t*526-*t*173	(II)-*t*526-*t*173	4, 5
6, 7	*su*105-*su*113-(I)	*su*105-*su*113-(VI)	9
1, 2, 3	(IV)-*t*526-*t*173	*su*105-*su*113-(I)	7
9	*su*105-*su*113-(VI)	*t'*106-*t*76-(VI)	8

TABLE 6

DEMONSTRATION OF STRONG NEGATIVE INTERFERENCE

Cross	Frequency of su^+t^+ recombinants
t′205 × *su*32	1.1×10^{-4}
*su*14 × *su*32	1.2×10^{-5}
*su*14-*t*′205 × *su*32	4.3×10^{-6}

shown by data in Table 6 derived from 2- and 3-factor crosses between three markers whose order is known from Table 3 to be *su*14-*su*32-*t*′205. Since the formation of wild-type recombinants in the cross *su*14-*t*′205 × *su*32 requires two exchanges, if these exchanges occurred independently of each other, wild-type recombinants would be expected to occur at a frequency of $1.1 \times 10^{-4} \times 1.2 \times 10^{-5} = 1.3 \times 10^{-9}$. It can be seen, however, that the observed frequency is 3300 times higher and is, in fact, almost equal to the lower of the 2-factor recombination frequencies. Not only are double recombination events frequent, but, as will be shown in the *Discussion*, for S13 they may be the rule.

Discussion.—The order of genes on the genetic map of S13 deduced in the present study agrees with the partial order deduced by E. S. Tessman[8] on the basis of 2-factor crosses. At the time of the original reporting of the complementation groups of S13, only five genes had been found and their order was reported as I-IIIa-IIIb-IV (as partly defined by the markers *t*526-*t*173-*su*43-*t*266-*t*330, in that order), with group II behaving as if it was distant from the other four genes. This mutual confirmation of 2- and 3- factor crosses supports the validity of the mapping experiments reported here.

For ϕX174, Pfeifer[26] found that 3-factor crosses gave segregation ratios close to 1:1 and concluded that either the genetic map was circular or recombination in this system was characterized by strong negative interference; the S13 results show that both obtain. It is quite possible that the strong negative interference is a simple consequence of recombination between circular DNA molecules since the mating between circular genomes is limited to an even number of exchanges if recombinant genomes of normal length are to emerge. In fact, the segregation ratios observed in the 3-factor crosses provide semiquantitative evidence that double recombination events are the rule for S13. Since the genome is comprised of approximately seven genes,[24] segregation ratios of about 6:1 are expected for an outside marker in an adjacent gene if double recombination is the rule. These are just about the ratios actually found. Even if S13 recombination events were not intrinsically correlated, there would still be strong negative interference because of the small number of matings experienced by individual phage particles.[27] Since markers approximately 180° apart on the genetic map recombine at a frequency of less than 1 per cent, it appears that only about 1/100 of the phage population undergoes heterologous mating. As a result, if a second recombination event occurs during these rare matings, its effect is exaggerated at least 100-fold.

The circularity of the map shows that recombination cannot always involve opening of the circular DNA at a unique site, although this does not rule out the possibility of such a unique site being involved in replication.

With the discovery of the seventh gene of S13, it was estimated[24] that 70–100 per cent of the genes of the phage have been detected by mutation. Thus, the genetic map reported here possibly represents a complete map of all the genes of this biological system.

Summary.—The seven known genes of S13 have been ordered by 3-factor crosses

into a circular genetic map. It is likely that most, if not all, of the genes of the phage are included. S13 recombination events exhibit a very high positive correlation, suggesting that possibly all genetic exchanges occur in pairs, which would conform with a requirement for genetic recombination between circular genomes. The circularity of the map rules out the possibility that recombination always involves opening of the circular DNA at a unique site.

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[1] The phage has a diameter of 250 Å[2] and contains single-stranded DNA[3-5] having a molecular weight of 1.7×10^6 daltons[5] (equivalent to approximately 5000 nucleotides) as measured in the closely related[6, 7] phage ϕX174.

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