GENETICAL TRANSFORMATION OF T4 PHAGE

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ADVANCES in a bacteriophage study will be reported here that supply us with additional information concerning (a) the mechanism of transformation, and (b) the nature of the infectiveness of phage DNA.

Some methodical problems of phage transformation: The occurrence of wild-type phage in a system consisting of *E. coli* B sphaeroplasts + T4*rII*⁺ DNA + π -factor² was considered by VAN DE POL, VELDHUISEN and COHEN (1961) to be the result of a genetic recombination between a phage chromosome and the DNA introduced into the system. Thus a biological activity of purified DNA introduced into the sphaeroplasts was considered to have been demonstrated (VAN DE POL *et al.*, 1961; VELDHUISEN *et al.*, 1962).

VAN DE POL's effect and the technique of his work are not in question, but, in our opinion, his choice of *rII* 51 and *rII* 35 mutants was not a very fortunate one, because they gave revertants, which could be easily confused with the transformants. For final proof that the T4 phage transformation does exist, we considered the use of nonreverting *rII* mutations that were deletions to be expedient.

We tried to reproduce VAN DE POL's experiment using the T4 phage mutant *rII* 250 as a recipient. The mutant *r* 250 as well as the other phage deletion mutants being used, were made available through the courtesy of PROFESSOR S. BENZER. The mutant is known to be situated in the *A* cistron of the *rII* region and is considered to be a deletion, after BENZER (1955, 1957, 1959, 1961). π -factor is obtained by urea treatment of the phage. The titer of the residual phage after treatment is about 10 to 10³ particles per ml. For the production of sphaeroplasts the penicillin technique is used as described in the literature (DENIS and POLGAR 1959; MARTIN 1963). Sphaeroplast production was controlled by means of anoptical phase contrast microscopy. DNA was prepared by the phenol technique after 0.1 M ethylenediamine tetraacetic acid (EDTA), and 6% para-amino-salicylic acid (PASA) treatment of high titer donor-phage samples. (MARMUR 1961).

The experiments have been carried out as follows: DNA is added to the sphaeroplast suspension and after careful mixing the π -factor is added. Each constituent of the system is taken in a volume of 1 ml. As a control we used a mixture of sphaeroplasts (1 ml), π -factor (1 ml), and instead of 1 ml of DNA 1 ml of Hottinger's broth³ containing 20% sucrose was taken. The detection and evaluation of T4 *rII*⁺ particles were made by plating the samples from the incubated system onto *E. coli* K lawns, and the initial phage is estimated by plating it upon *E. coli* B lawns.

Since it was determined that the number of *rII*⁺ particles is the same for a concentration of 490 μ g/ml as for 49 μ g/ml DNA introduced (Figure 1), an

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² Here, a urea treated *rII* 51 T4 phage.

³ Tryptic digest of meat used instead of tryptone (GOLDFARB and TIMAKOV 1958).

FIGURE 1.—The effect of DNA concentration on the yield of *rII*⁺ transformants.

experiment has been carried out to determine the time effect of DNA introduction into the system. The results of the four variants of this experiment are shown in Table 1.

It can be seen from the data that a maximal effect was obtained in the case of preincubation of sphaeroplasts with DNA (Variant 1); the simultaneous introduction of π -factor and DNA, and the introduction of DNA 10 minutes after π -factor addition (Variants 2 and 3) gave fewer transformants. No *rII*⁺ plaques are detected when DNA is introduced into the system after 90 minutes incubation (Variant 4). Therefore, all further experiments have been carried out with 30 min preincubation of DNA with sphaeroplasts.

The influence of the concentration of π -factor on the *rII*⁺ phage yield is shown in Table 2. The table shows that the optimal proportion of *rII*⁺ phages depend on the π -factor concentration.

In our experiments it was of interest to study the comparative development of *rII*⁺ and *rII* phages. For this purpose *rII* 250 π -factor is introduced into the system after preliminary incubation (30 min) of *rII*⁺ DNA with the sphaeroplasts. For the evaluation of mature phage particles, the chloroform treated samples are

TABLE 1

The time effect of DNA introduction into the system on the yield of T4 rII⁺ phage

Variant No.	Time of sampling	Centres of infection	Mature phage
1	10 minutes	8.5	0
	3 hours	410	480
2	10 minutes	8.0	0
	3 hours	160	180
3	10 minutes	5.0	0
	3 hours	130	160
4	3 hours	0	0

TABLE 2

The effect of π -factor concentration on rII phage yield

π -factor concentration	Plaque number on	
	<i>E. coli</i> K	<i>E. coli</i> B $\times 10^6$
10-fold condensed	30	3.8
2-fold condensed	69	3.3
Original sample	86	3.4
2-fold diluted	180	1.7
5-fold diluted	430	2.1
10-fold diluted	100	1.5
20-fold diluted	50	1.2

plated upon *E. coli* B and K, and for the evaluation of infective centres the samples without treatment are plated on the same hosts. As can be seen from Figure 2, the number of infective centres (*rII*⁺) remained unchanged up to the 30th minute, then gradually increased, and reached its constant level by the 60th

FIGURE 2.—Comparison of the *rII* 250 recipient phage and *rII*⁺ transformants. Upper curves (thick lines): ○—centres of infection, *rII* 250; ×—mature phage, *rII* 250. Lower curves (thin lines): ○—centres of infection, *rII*⁺; ×—mature phage, *rII*⁺.

minute, and its yield grows in parallel to an increase in the number of infective centres. The increase of the *rII* 250 infective centres and a concomitant increase of *rII* 250 mature phage yield can be seen already by the 20th minute of incubation. Thus, 3 hours incubation is sufficient for the completion of phage development in the system.

Experiments have been carried out to study the effect of DNA introduction into the system without DNase treatment, and the effect of DNase on the activity of DNA, when DNA was pretreated with DNase during 1 hour at 37°C before introduction. In the first case, 95 *rII* particles per ml were observed; in the second case, no *rII* particles have been demonstrated.

In an experiment concerning DNase effect on DNA activity after the incubation of the DNA with the sphaeroplasts, the following two variants of DNase addition were tested: (1) after preincubation of DNA with the sphaeroplasts, DNase was added, the suspension was then incubated 1 hour at 37°C, and the π -factor was introduced; (2) DNase was introduced either simultaneously with the π -factor or 10 min following its addition. In all cases yields of the order of 100 per ml have been obtained. When DNA, π -factor, and DNase were added simultaneously to the sphaeroplasts without preincubation, 4000 phages per ml were found. It follows from the data that DNase completely disrupts the DNA activity only in the case of its pretreatment apart from the system.

To clarify the specificity of DNA action, two experiments were carried out with the introduction of DNA isolated from *B. subtilis* and DNA from T4 *rII* 250 phage. In both cases no *rII*⁺ phages have been detected.

Thus, the results of VAN DE POL *et al.* were confirmed, and the methods for the determination of the biological activity of DNA isolated from T-even phages were further developed. Now we can be certain that *rII*⁺ phages obtained in our experiments are not the spontaneous revertants of *rII* mutants. It was convincingly demonstrated that phage DNA of *rII*⁺ type goes into sphaeroplasts, and only after entering does it recombine with phage DNA. It is clear from the data that the rate of DNA penetration into sphaeroplasts is rather high, because the simultaneous introduction of DNA and DNase into the system does not prevent the production of *rII*⁺ phage.

A demonstration of the transformation effect when the intact T4 rII phage is used

As is well known, in penicillin induced sphaeroplasts only a part of the cell wall is substituted by the plasmatic membrane, through which the DNA molecule penetrates more easily. BASS and ILJASHENKO (1959) in their experiments have shown that an intact valid phage could be adsorbed on the remaining regions of the cell wall. The question arises whether the system is capable of working without the π -factor, i.e., whether it is possible to transform an intact recipient phage, which is supposed to infect the sphaeroplast via remaining cell wall pieces, while DNA is going through the protoplasmic membrane of a sphaeroplast.

As a recipient phage, we used mutant T4 *rII* 1272, which is a large deletion involving the

TABLE 3

Determination of the optimal sphaeroplast/phage ratio

Sphaeroplast/ phage ratio	Frequency of <i>rII</i> ⁺ transformants with the sphaeroplast titer		Recipient phage <i>rII</i> 1272 yield, with the sphaeroplast titer	
	10 ⁷	10 ⁸	10 ⁷	10 ⁸
1 : 0.1	1	2	7.0×10^6	6.3×10^7
1 : 1	2	1		
1 : 5	2	4		
1 : 10	1	5		
1 : 50	16	24		
1 : 100	78	39	6.0×10^7	1.5×10^9

entire *A* and *B* cistrons of the *rII* region; in other words, it is a deficiency of a chromosome fragment larger than the *rII* 250 deletion used in previous experiments.

Since it is supposed that the phage can infect a sphaeroplast only by being adsorbed on the remaining cell wall pieces, the proper determination of the phage/sphaeroplast ratio is of importance. For this purpose, DNA is added to the 1.0×10^7 and 1.0×10^8 sphaeroplasts per ml, and after 30 minutes of preincubation the phage is introduced into the system in the following proportions: 0.1, 1.0, 5.0, 10.0, 50.0 and 100 phages per sphaeroplast. After 3 hours incubation of the system, the samples were plated to reveal the infective centres of the recipient phage on the *E. coli* B lawn, and of the transformants on the *E. coli* K lawn.

The experimental results are shown in Table 3. It follows from the data, that the most favourable ratio for the burst of transformants is 100 phages per sphaeroplast. It is important to note also, that the number of infective centres of the recipient *rII* 1272 phage increases 6 to 7-fold for the ratio 1:0.1 (in comparison with the phage introduced— 1×10^6 and 1×10^7), which indicates that phage development occurs inside the sphaeroplasts. When 100 phages per sphaeroplast are added to the system, a decreased number of infective centres of the recipient *rII* 1272 phage is observed relative to the introduced phage number, which indicates, in our opinion, the occurrence of lysis-from-without.

It is necessary to take into consideration the fact that an absolute increase of introduced sphaeroplasts (and therefore of phage) does not lead to an increased yield of transformants. The number of transformations increases only when the system containing 10^8 sphaeroplast per ml and 10^{10} phages per ml is incubated

TABLE 4

The "dilution effect" of a system on the number of transformants

T4 <i>rII</i> ⁺ DNA + recipient phage	Number of transformants per ml of undiluted system	Sum of ten samples of a diluted system
<i>rII</i> 1272	31.5	215.5
<i>rII</i> 386	53.5	430
<i>rII</i> PT 153	26.5	465
<i>rII</i> H 88	16	415
<i>rII</i> 184	9	1098.5
<i>rII</i> 250	36	504
<i>rII</i> 164	17.5	262

10 min at 37°C, diluted 10-fold in Hottinger's broth with 20% sucrose, incubated again for 3 hours, and plated on *E. coli* K (ten plates with a volume of 1 ml per plate). The "dilution effect" has been reproduced for the number of other recipient phages. Table 4 shows the results of these experiments.

It is clear from the data that the number of infective centres for the plates with diluted samples is always greater (in a sum of ten dishes) than with undiluted samples. Such an increase of the number of infective centres in diluted samples is observed only for the *rII*⁺ transformants; such an effect is not detected for the recipient phage.

*The use of T4 phage transformation for the genetical analysis
of rII region deletions*

BENZER (1955, 1957, 1959, 1961) by a large-scale crossing of mutants has mapped in detail the *rII* region of T4 phage and has evaluated the sizes and location of these deletions.

We have carried out experiments for establishing transformation in phages having deletions of differing size and topology inside the *A* cistron, and for the accomplishing cross-transformation between different deletions, i.e., inside the system, where both recipient and donor phages are deletions.

It was supposed here that transformations should be produced only in cases where the deletions of donor and recipient phage DNA did not overlap, according to BENZER's map. Seven phage strains—1272, 386, PT 153, H 88, 184, 250, and 164—with deletions in the *rII* region of cistron *A*, were used. As one can see from Figure 3, these deletions are of different sizes. Thus, mutant 1272 is a deletion involving both *A* and *B* cistrons; deletion H 88 occupies a large part of

FIGURE 3.—Localization of different deletions in the *A* and *B* cistrons of the *rII* region of the T4 phage chromosome (after BENZER 1964).

TABLE 5

The transformation effect of wild phage T4 DNA on different deletions

	Recipient phages						
	1272	386	PT 153	H 88	184	250	164
T4 <i>rII</i> + DNA	320	500	610	800	1400	810	450

cistron *A*. The other deletions investigated are smaller ones, and occupy various positions inside the *A* cistron.

Before carrying out cross-transformations, it was necessary to elucidate whether the transformation of all the deletions could be produced by wild-phage DNA. The resulting experiments are collected in Table 5.

The data show that wild-phage DNA is effective in transforming all the deletions studied. The data suggest that the number transformed is dependent on the size of the deletion used. These data, however, are not sufficient for a final conclusion about the linear dependence of the number transformed upon the size of the transformed deletion, since the transformation rate of the largest deletion, 1272, is smaller than those of mutant phages with smaller deletions.

The cross-transformation experiments were carried out by introducing to the system a specific mutant phage plus DNA isolated from another mutant, and vice versa. As a control, wild-phage DNA was introduced into all the mutant phages. The results are shown in Table 6. Values given in the body of the Table are the proportion of transformants obtained using DNA from a mutant phage, relative to the transformants in the control (i.e., when the same mutant phage was being transformed by DNA isolated from wild phage).

For better illustration, the data obtained for the seven deletions studied are expressed graphically in Figure 4, where the mean numbers of reciprocal experiments are used, from Table 6. Deletion 386 has been taken as an initial point, and the distances of the others are determined according to the transformation data between separate deletions. As one can see, the location of deletions relative to each other, and the deletion size as well, coincide with the *A* and *B* cistrons of *rII* mapped by BENZER on the basis of mating the deletion strains (Figure 3). The

TABLE 6

The results of cross-transformation for the two reciprocal variants

Phage	Source of transforming DNA						
	1272	386	PT 153	H 88	184	250	164
1272	0	0	0	0	0	0	0
386	0	0	3.8	7.8	7.3	15.0	22.3
PT 153	0	1.3	0	0	0	0	7.5
H 88	0	2.1	0	0	0	0	0
184	0	13.1	0	0	0	0	8.2
250	0	10.4	0.36	0	0	0	40.0
164	0	14.0	6.2	0	8.8	7.8	0

FIGURE 4.—Localization of different deletions in the *A* cistron of the *rII* region, according to the cross-transformation data from Table 6.

only exception is the localization of deletion pair PT 153–184, as was noted earlier. The position of deletion 146 according to the transformation data should be the same as in Figure 3 (dotted line for 386, and solid line for 250), when the partners were 386 and 250.

The data show that cross-transformation could not be produced in the following cases: (1) When the recombining chromosome and the DNA have deletions completely overlapping each other, for example in the case of 1272, which is longer than any other deletion used, or when H 88, 184, and 250 are taken, which overlap one another. (2) When the deletions only partly overlap one another, for example PT 153–H 88 and H 88–164. It seems clear from the data that transformations have been realized only in the cases where non-overlapping mutant phages were used. In those cases, the number of transformants obtained depends on the map distance between the deletion pair used. It should be noted that no transformation was detected between PT 153 and 184, deletions situated very close to each other, and there was a small amount of transformation between the neighboring deletions PT 153 and 250 in the only experiment in which 250 mutant phage and PT 153 phage DNA were added to the system.

All the experiments reported here were repeatedly reproduced with similar results.

DISCUSSION

The T4 chromosome deletions obtained and described by BENZER (1961) are a very convenient object for the study of transformation in phages, since spontaneous reversion to the wild type is excluded, and the results give a more precise basis for examining the transformation mechanism.

It was shown that the transforming effect of T4 phage could also be obtained without urea pretreatment of the phage, but only if there was a steep rise of phage number per sphaeroplast (in our case, 1:100), which increases the probability of phage adsorption upon the remaining islands of the sphaeroplast cell-wall. This

suggests a possible method that would allow the introduction of more strict quantitative control during phage transformation studies and would exclude the necessity of the additional urea treatment of the phage.

The so-called "dilution effect" demonstrated here attracts one's attention, and at the same time the qualitative differences obtained with various mutants are of some interest. These phenomena are under investigation.

On a basis of our data, VAN DE POL's effect could be interpreted as a transformation initiated by phage DNA. The effect obtained could be explained as a recombination between a mutant phage chromosome and either wild phage DNA or mutant DNA. Since rather large deficiencies of a genetic structure (such as the 1272 deletion) can be repaired by means of transformation, one may assume that the DNA molecule penetrating into a sphaeroplast is large enough to overlap the deficiency region.

The lack of a strong correlation between the number transformed and the size of transformed deletion also favours the supposition that the size of the transforming DNA molecule is considerably greater than the sizes of the deletions studied. Most convincing evidence comes from the cross-transformation experiments, where a correlation has been obtained between the number transformed, on one hand, and the distance between the deletions, on the other. This confirms the supposition, that recombination between a chromosome and DNA molecule is the basis of genetic transformation. We consider the samples of transforming DNA introduced into the system to consist of parts of DNA molecules longer than the extent of the *rII* region under investigation. One may admit that these DNA fragments recombine with a certain frequency with a recipient phage chromosome, and therefore are under the recombination laws observed in phage crosses.

In the case of the PT 153-184 pair, there were no transformants detected, although the deletions, according to BENZER's data, did not overlap each other (Figure 2). Presumably recombination in this case is of such a low frequency that we could not detect it.

These data indicate that the transformation process may plausibly be used for biochemical studies of T4 phage DNA activity in connection with its genetical functions. Finally, they should be considered as our first approach to solving the problem of the infectiveness of DNA isolated from T4 phage.

SUMMARY

For studying transformation of T4 phage, deletions of the *rII* region obtained by S. BENZER were used. The possibility was thus avoided of confusing transformation with spontaneous mutation. Purified DNA isolated from wild phage was used for transforming.

Transformation was initially studied in a system: sphaeroplasts + π -factor + DNA. It was shown that transformation does occur in the *rII* region. Transformation has been further shown to be accomplished also without urea pretreatment of the phage, which permits more precise investigation of the phenomenon.

The number of transformants obtained depends on the dilution of the system (the "dilution effect"); this is, however, so far unexplained.

The results of cross-transformation of different deletions permits mapping them within the *rII* region using a new method.

Mapped so, the deletions coincide with the locations made earlier by BENZER on the basis of matings.

Резюме

Для исследования трансформации ффага Т4 использовались делеции *rII* области, полученные С. Бензером. Тем самым исключалась возможность подмены эффекта трансформации спонтанными мутациями.

Трансформация изучалась первоначально в системе: сферопласты + λ -фактор + ДНК. Было показано, что трансформация делеций *rII* области действительно имеет место. Далее было показано, что явление трансформации можно осуществить без предварительной обработки ффага мочевиной, что позволяет точнее исследовать это явление. Обнаружена зависимость числа полученных трансформантов от разведения системы ("эффект разведения"). Однако, сущность его пока не поддаётся объяснению.

Результаты перекрёстной трансформации различных делеций позволили локализовать их с использованием нового метода на карте *rII* области. Локализация делеций при таком картировании совпадает с их расположением, обнаруженным ранее Бензером при помощи скрещиваний.

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