TRANSFORMATION IN PHAGE T4: MINIMAL RECOGNITION LENGTH BETWEEN DONOR AND RECIPIENT DNA

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DNA-mediated transformation of genetic characters has been used extensively as a tool in the search for an understanding of the recombination process at the molecular level. The combined evidence obtained through genetic and biochemical studies has divided the sequence of events in DNA transformation into three clearly defined steps: (1) uptake of transforming DNA by competent recipient cells; (2) pairing of the immigrant DNA molecules with homologous regions of the recipient genome; (3) integration of the donor DNA into the genome of the recipient.

Whereas step (1) is a specialized feature of transport of a macromolecule through the cellular envelope and not related to the problems encountered in conventional studies on genetic recombination, steps (2) and (3) are likely to be very similar to, if not identical with, the two final steps in recombination.

It is thought that pairing of donor with recipient DNA requires the recognition of complementary nucleotide sequences in the two DNA molecules. Studies on DNA mediated transformation in bacteria have indicated that the smallest DNA fragment still capable of transforming a mutant marker has a molecular weight of approximately 1×10^6 (LITT, MARMUR, EPHRUSSI-TAYLOR and DOTY 1958; ROSENBERG, SIROTNAK and CAVALIERI 1959), corresponding to approximately 1,000 base pairs. This size may not, however, represent the minimal recognition length required for the successful lining up between donor and recipient molecules, since a minimal size of the donor DNA may be required for either its uptake or its function as a substrate for the enzymes effecting its integration into the recipient genome. In this latter case, the size of the donor DNA fragment found in the recipient DNA after integration (Fox 1962) is not necessarily identical with the length of the nucleotide sequence involved in the recognition process between the donor and recipient molecules.

In this paper we describe a series of experiments using the T4 transformation system for studies on recombination which were designed to obtain an estimate of the minimal sequence homology required in the recombination process. The results led us to the conclusion that the recognition of two homologous sequences requires only a small number of nucleotides.

MATERIALS AND METHODS

Bacterial and phage strains: E. coli strain B was used in all experiments for the formation of

spheroplasts by the penicillin method (GOLDBERG 1966). As indicator bacteria in a transformation experiment *E. coli* strains B and K-12 (λ) were used. Since all T4 phages having a mutation in the *rII* gene are unable to grow on K-12 (λ), this indicator could be used selectively for the detection of r^+ recombinants. The *rII* deletion mutants used as DNA sources and recipient phages are schematically presented in Figure 1. A double deletion mutant was constructed by a cross between the *rII* deletion mutants *r*1631 and *r*Pb28.

Media and other solutions: The phage stocks were grown in a glucose-salts medium which consists of (in mmoles) 50 Na_2HPO_4, 22 $KHPO_4$, 19 NH_4Cl, 1 $MgSO_4$, 28 dextrose and 0.005 $FeCl_3$. The media used for the preparation of spheroplasts and all other solutions have been described previously (BAUTZ 1966).

Preparation of donor DNA: The method of GOLDBERG (1966) was used.

Fragmentation of DNA: In order to produce DNA fragments of approximately uniform length, native DNA was sonicated in a Mullard Ultrasonic Disintegrator at a concentration of 20 μg/ml for the times indicated. The same DNA concentration was used for the denaturation process, which was carried out in 0.01 M phosphate buffer (pH 7.0) by heating the DNA 5 minutes at 95°C and chilling rapidly.

Molecular weight measurement of DNA fragments: Samples of native T4 r^+ DNA (20 μg/ml) in 0.01 M phosphate pH 7.0 were subjected for 5, 20, and 40 seconds to the maximal output of a Mullard Ultrasonic Disintegrator. The sedimentation coefficients for these DNA samples were determined by sedimentation in a Model E ultracentrifuge at 20°C and are: 24.6S, 11.6S and 9.8S, corresponding to a molecular weight of approximately: 12×10^6, 2×10^6 and 1×10^6 respectively.

Transformation reaction mixture: E. coli B spheroplasts (0.5 ml) and 4 μg/ml DNA (0.5 ml in 0.01 M phosphate pH 7.0) were mixed. After incubation at 37°C for 15 min 0.05 ml of T4 *rII* helper phage (1×10^{12}) was added and the mixture was incubated for 3 hr at 37°C after which chloroform was added.

Helper phage: Urea treated helper phage which infect *E. coli* spheroplasts produced by penicillin treatment were prepared by the method of GOLDBERG (1966).

Correlation between physical and genetic map distance: The *rII* region in phage T4 can be

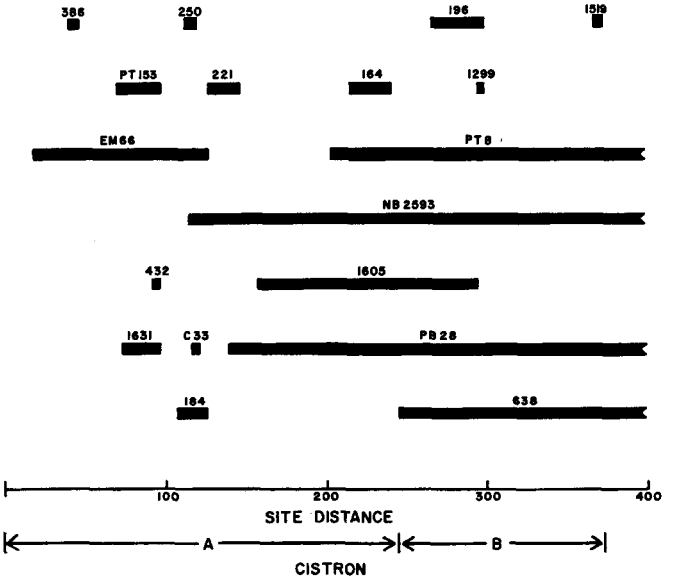

FIGURE 1.—Map positions of *rII* deletion mutants used. Map distances are given in site numbers as defined in MATERIALS and METHODS.

divided into 373 individual mutation sites (BENZER 1962). Independently isolated point mutants which fail to recombine, are considered to occupy identical sites on the *rII* map. The size of the *rII* region is estimated at 1% of the total T4 genome, which is equivalent to some 2,000 base pairs. Assuming a random distribution of mutant sites, the physical distance between two sites is equivalent to 2,000/373—about five base pairs. Marker distances are usually given as mutation sites.

Experimental design: DNA obtained from a mutant phage carrying an *rII* deletion was used to transform the *rII* character of helper phages carrying deletions in the *rII* region at various distances from the deletion in the donor DNA. This is analogous to a phage cross between two *rII* mutants, and an example of such a cross is shown in Figure 2. The helper phage carries a deletion to the left of *c*, opposite to loop *b* in the donor DNA; likewise the donor DNA carries a deletion opposite loop *d* of the helper phage. In one set of experiments the same donor DNA is crossed with different helper phage mutants, thus the distance *c* is varied.

Transformation requires two independent exchanges x' and a' for the production of an $r+$ phage genome. The frequency of obtaining an $r+$ recombinant is then:

$$r = \int_0^c dx \int_0^{L-(b+x)} da = \int_0^c dx\ [L-(b+x)] = (L-b)c-c^2/2 \qquad (1)$$

Dividing both sides by c yields:

$$r/c = (L-b) - c/2 \qquad (2)$$

Plotting the yield of $r+$ transformants as r/c versus c one should obtain a straight line with a decreasing slope as the distance c between the two deletions increases.

The rationale for using the T4 DNA transformation system rather than performing conventional phage crosses was that one of the two marker genomes was present in the form of a small DNA fragment, allowing c to be varied strongly with regard to $L-b$ and, more importantly, minimizing interactions of outside regions, as indicated by the double arrows of Figure 5.

RESULTS

Figure 3 shows the results of a number of crosses, done as outlined above, between denatured (3a) or native (3b) donor DNA and *rII* helper phages, isolated from several *rII* deletion mutants. If sonicated denatured DNA is used as the transforming principle, the ratio r/c decreases as the distance c between the two marker increases. The steepness of the slope suggests that the average DNA fragment incorporated is equivalent to some 100 to 150 mutation sites or 500 to 750 nucleotides. In contrast, native DNA does not show the decreasing slope as expected from equation (2), indicating either that native DNA is much larger than the longest marker distance tested ($c \ll (L-b)$), or that native DNA does not become integrated like denatured DNA, but is functionally equivalent to an in-

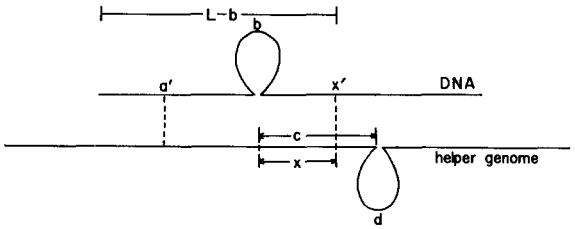

FIGURE 2.—Schematic outline of a cross between transforming DNA and helper phage genome, each carrying an *rII* deletion. L: the length of the polynucleotide chain to the left of x', b: the number of nucleotides missing from the helper genome, c: the number of nucleotides missing from the donor DNA.

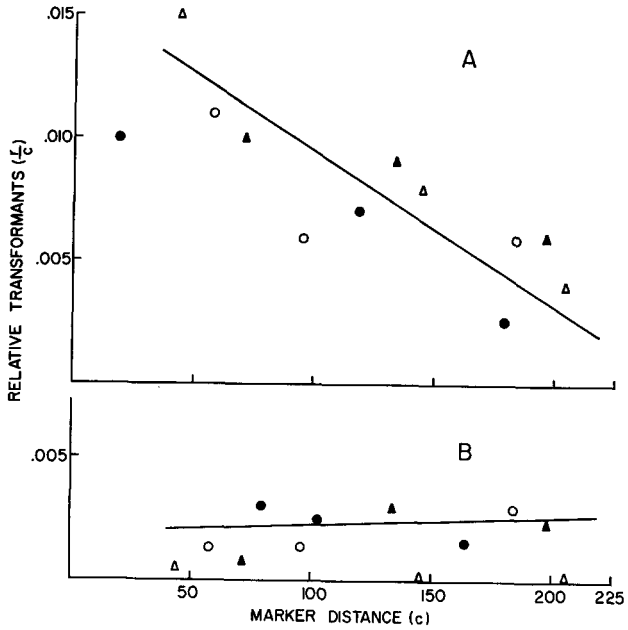

FIGURE 3.—Determination of the number of transformants relative to marker distance (r/c) for native and sonicated denatured DNA isolated from different mutants carrying deletions in the *rII* genes. Figure 1A shows the r/c values observed for denatured DNA sonicated for 10 seconds △: *r*1299 DNA and helper phages *r*164, *r*250, *r*386. ●: *r*196 DNA with helper phages *r*164, *r*250, *r*386. ○: *r*221 DNA with helper phages *r*164, *r*196, *r*1519. ▲: *r*EM66 DNA with helper phages *r*164, *r*1299, *r*1519. Figure 1B represents the number of relative transformants for native DNA: △ *r*1299 DNA with helper phages *r*164, *r*1299 and *r*1519. ●: *r*638 DNA with helper phages *r*221, *r*250 and *r*386. ▲: *r*EM66 DNA with helper phages *r*164, *r*1299 and *r*1519. ○: *r*221 DNA with helper phages *r*164, *r*196 and *r*1519.

complete genome, requiring one crossover event only (at x', Figure 2). In this case, r would equal $\int_0^c dx$, i.e. the ratio r/c should be a constant for all values of c.

The average segment of DNA integrated into the genome of the helper phage may thus be relatively small for denatured DNA and large for native DNA. This suggestion is supported by the observation that native DNA rapidly loses its transforming activity upon sonication whereas denatured DNA becomes slightly more active if a few breaks are introduced. Native DNA differs from denatured DNA in another respect: whereas optimal yields of transformants are obtained with 2 μg of denatured DNA, with native DNA the yield of r^+ transformants increases almost linearly up to 100 μg of DNA per ml of incubation mixture.

It is evident from the decreasing slope in Figure 3a that r/c should be highest in crosses between very close deletions (i.e. for very small c). However, if a crossover event requires the recognition of homologous nucleotide sequences, one might expect a recognition failure as the distance c becomes too short. Crosses between denatured DNA and helper phages of *rII* deletions less than 20 (for helper *r* 432) or 35 (for helper *r*PT153) mutation sites apart yielded r/c values which no longer follow the slope obtained from crosses between deletions located

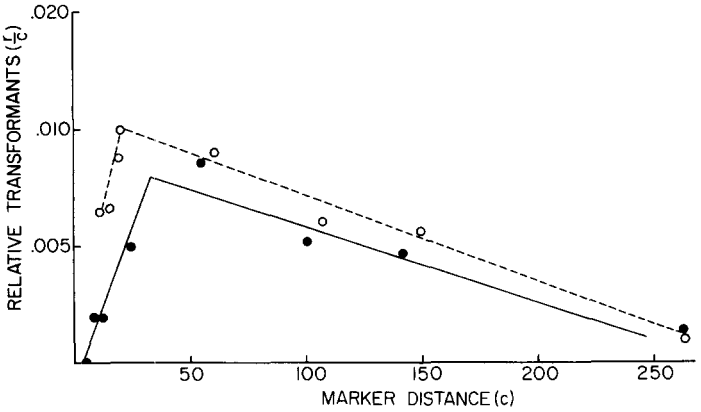

FIGURE 4.—Correlation between minimal site distance of two markers and maximal yield of transformants for 10 seconds sonicated denatured DNA isolated from different *rII* mutants. The ratio r/c was plotted versus c as in Figure 3. ●: Helper phage *r*PT153 with DNA from: *r*184, *r*250, *rNB*2593, *r*221, *r*1605, *rPT*8, *r*638, *r*1519. ○: Helper phage *r*432 with DNA from *r*184, *r*250, *r*NB2593, *r*C33, *r*1605, *r*PT8, *r*638, *r*1519.

further apart (Figure 4). This rather drastic drop in the yield of r^+ transformants indicates that the probability of a recombinational event between the two markers becomes suddenly reduced if the marker distance drops below a critical length. The ratio r/c approaches zero at a distance equivalent to a few mutation sites (approximately five in the case of helper phage *r*PT153 with DNA from *r*184). Whether this distance represents the minimal recognition length depends upon whether or not the lining up of donor and recipient DNA between loops *b* and *d* (Figure 2) is stabilized through the pairing of the homologous regions to the left of *b* and to the right of *d*.

In order to test the influence of outside regions on the recombination frequencies between close markers, we have studied crosses of the type outlined in Figure 5. Here, the transforming DNA carries two deletions requiring two recombinational events (x_1' and x_2') within two genetically well defined segments of the *rII* region. If the lining up between donor and recipient DNA at c_1 and c_2 is stabilized through pairing elsewhere, indicated by the double arrows, we

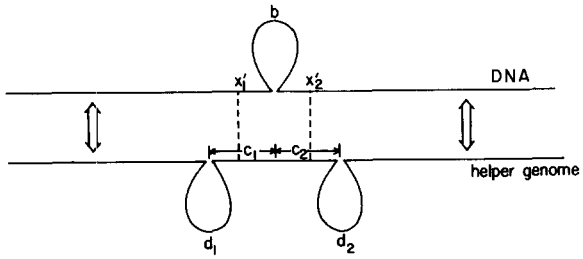

FIGURE 5.—Schematic outline of a cross between DNA carrying two deletions (opposite d_1 and d_2) and the helper phage genome, carrying a single deletion (opposite *b*). The double arrows indicate the possible contribution of homologous outside regions to the lining up at c_1 and c_2.

should expect the yield of r^+ transformants to be a function of the size of the donor DNA fragment. The longer the paired outside regions, the more they should help the pairing at c_1 and c_2. On the other hand, if such a helping effect does not exist, the yield of r^+ transformants should be independent of the size of the double mutant DNA over a wide range.

The double deletion used was constructed by crossing *r*1631, a small deletion located near the left hand end of the *rIIA* cistron, into *r*Pb28, a large deletion covering the right half of the *rIIA* and all of the *rIIB* cistron (BENZER 1961). The two deletions are a distance equivalent to 42 mutation sites apart. DNA prepared from a phage stock of this double deletion was then tested with helper phages carrying single deletions of slightly different sizes and map positions. Table 1 shows the helper deletions used, together with their location relative to the two deletions of the donor DNA. The last column gives the yield of r^+ transformants relative to the r^+ transformants obtained from crosses of the same helper phages with r^+ DNA. Helper *r*221 overlaps with *r*Pb28, its failure to yield r^+ transformants is expected and serves as a control.

In a subsequent experiment r^+ DNA and DNA from the double mutant *r*1631/Pb28 were sonicated for increasing length of time and used to transform the helper phage *r*250. The result of this experiment is shown in Figure 6 and suggests that *r*1631/Pb28 DNA shows no change in transforming activity when sonicated from 5 to 40 seconds, whereas r^+DNA decreases proportionally with increasing time of sonication. The insensitivity in transforming capacity of *r*1631/Pb28 DNA to fragmentation seems to indicate that the decrease in molecular weight of this DNA is not crucial up to a certain point since only a small piece of the *rII* region is physically present in this mutant DNA and recombination at c_1 and c_2 necessarily involves only a short DNA segment. This finding suggests that recognition of DNA sequences located beyond the deleted regions are not influencing substantially the yield of r^+ transformants.

DISCUSSION

The unexpectedly low yields of r^+ transformants observed in crosses between donor and recipient molecules carrying deletions less than the equivalent of 25

TABLE 1

Transformation of the r+ *character by a DNA fragment located between two* rII *deletions*

rII deletion (D_x) used as helper phage	Site distance			r^+ transformants*
	C_1	C_2	D_x	
*r*250	14	22	6	0.034
*r*C33	18	19	5	0.033
*r*184	10	14	18	0.010
*r*221	30	0	12	0

* Number of r^+ transformants obtained with *r*1631/Pb28 DNA divided by the number of r^+ transformants obtained with r^+ DNA.

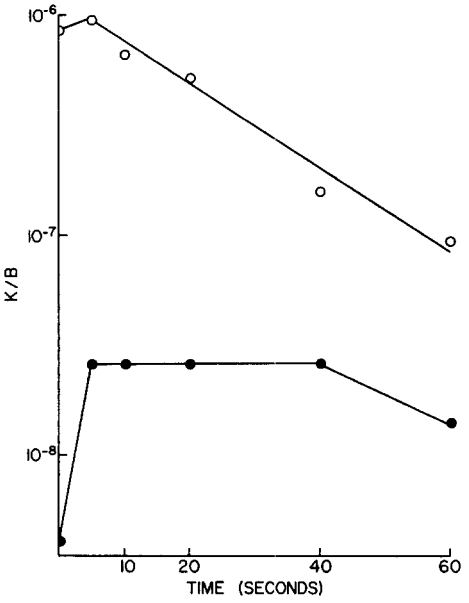

FIGURE 6.—Transformation experiment with helper phage *r*250 and denatured DNA isolated from r^+ and *r*1631/Pb28 phage stocks. The *K/B* values obtained with the two DNA samples sonicated for the times indicated were plotted logarithmically versus time of sonication. O: r^+DNA; ●: *r*1631/Pb28 DNA.

mutation sites apart (Figure 4) suggests that the mutual attraction of donor and recipient DNA between *b* and *d* (Figure 2) is not strong enough for integration to occur with normal efficiency. Whereas single base differences due to point mutations should exert only a negligible effect on the stability of a helix (FRESCO and ALBERTS 1960) and even permit recombination within a single codon unit (HENNING and YANOFSKY 1963), probably through lining up of homologous sequences on either side, it is likely that the two loops *b* and *d*, representing sizeable regions of no homology, prevent the proper lining up of donor and helper DNA in their vicinity. The interpretation that deletions have a disruptive effect on the pairing of neighboring regions of homology, is substantiated by the data of Figure 6, which show that the transforming capacity of a double deletion DNA for a marker located between the two deletions is insensitive to fragmentation if compared with wild-type DNA. In the absence of any cooperative effect, the smallest marker distance to produce a finite yield of r^+ recombinants can be regarded as the minimal recognition length required for the successful lining up of two complementary sequences. Its size can be roughly estimated as the equivalent of five mutation sites or approximately 25 base pairs.

A small minimal recognition length applies rather severe restrictions to the distribution of nucleotides within a freely recombining genome (a genome not restricted by compartmentalization or position effects to undergo recombination with any part of itself or a related genome) in that no DNA sequence equal to or longer than the minimal recognition length may occur more than once (THOMAS 1966), or else it would be rapidly eliminated through either internal or unequal crossing over. Theoretically, the minimal recognition length could involve as short a sequence as ten nucleotides in the case of phage T4 or 12 nucleotides in the case of most bacterial genomes. If natural selection were to favor nonrepe-

titious sequences, all decanucleotide sequences in the T4 genome would be occurring just once. Experimentally, fragments of *rII* specific mRNA of a size of 13 nucleotides have been found to hybridize specifically with DNA possessing the *rII region* (W. RÜGER and E. K. F. BAUTZ, in preparation) indicating that most sequences of this size are not recurring elsewhere in the T4 genome. The recognition of sequences of some 25 bases, as suggested by our results, should provide enough specificity to keep unequal crossing over, occurring as the result of nonhomologous pairing, at the extremely low level required for the proper functioning of the genetic machinery.

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SUMMARY

The T4 DNA transformation system, in which denatured as well as native DNA transform, was used to study recombination between *rII* mutations located on the donor and recipient genome. The frequencies of r^+ transformants resulting from recombination between DNA and helper phage carrying very closely linked markers were found to be much lower than those predicted from the slope obtained from crosses between more distant markers. This observation indicates that there is a critical distance required for effective lining up of donor and recipient DNA.—DNA of an *rII* double deletion mutant straddling the genetic marker of an *rII* helper phage was found to yield r^+ transformants with a characteristic frequency which was insensitive to fragmentation of the transforming DNA, whereas the yield of transformants by r^+ DNA was sensitive to fragmentation. This result suggested that pairing of outside regions had little, if any, influence on the yield of r^+ transformants.—The "critical recognition length", i.e. the number of nucleotides required for complementary base pairing between donor and recipient DNA molecules, was estimated at roughly 25 nucleotides.

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