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Tandem Duplication in Bacteriophage P2: Electron Microscopic Mapping

(heteroduplexes/novel-joint/physical and genetic maps/P2 *vir37*)

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ABSTRACT **The physical position of *vir37*, a new immunity-insensitive mutant of *Escherichia coli* bacteriophage P2, was mapped by the electron microscopic heteroduplex method. In P2 *vir37*, a segment equivalent to 2.8% of P2 DNA is added. The addition was characterized as a tandem duplication of the segment occurring between 77.2 and 80.0% from the left end of P2 DNA (the right half of P2 DNA is arbitrarily defined, from denaturation map studies, as the half richer in A + T). The point of addition of the duplicated segment (the "novel-joint") was, thus, 80.0% from the left end of P2 DNA. On the basis of previous studies on P2 *vir22*, it was tentatively concluded that the physical and genetic maps of P2 are colinear. This conclusion is now further supported by physical and genetic data on P2 *vir37*.**

In the electron microscopic heteroduplex method (1, 2), nonhomologous regions of DNA are visualized as single-stranded loops of unique size and location in otherwise double-stranded molecules. One exception to this occurs with addition mutants when the added segment is a tandem genetic duplication; here the loop position is variable. The movable loop should show the following properties: (*a*) loop size remains constant although its position may vary, the complementary strand remains fully base paired with the possible exception of a few unpaired bases at the junction of the loop with the double helix; and (*b*) the loop could move over a length equal to that of the duplicated DNA segment. Also, tandem duplication mutants should segregate in a recombination-proficient host as a series of phages whose DNA lengths differ from the next by an amount equal to the primary DNA addition. Such properties have recently been demonstrated in a derivative of bacteriophage λ (3, 4). In the present study, similar evidence will be presented for a tandem duplication in DNA from P2 *vir37*, a new immunity-insensitive mutant of temperate *Escherichia coli* phage P2 (5).

MATERIALS AND METHODS

The following phages were used in the present study: P2 *vir37*, P2 *lg cc vir22*, and *P2 lg del1*. *vir37* is a new mutation (5) which, like *vir22*, confers immunity insensitivity. Phage preparations, or samples of their DNA, were supplied by G. Bertani. For brevity, P2 *lg cc vir22* will be referred to as P2 *vir22* and P2 *lg del1* as P2 *del1*.

Heteroduplexes were formed, following the method of Davis and Parkinson (7) but on a reduced volume. Renaturation (final volume of the mixture, 0.2 ml) was carried out 23° for 1 hr in a small conical polystyrene vial (Evergreen Scientific, Los Angeles). The sample was subsequently dialyzed against 0.02 M NaCl–0.005 M Na_2EDTA (pH 7.4) at 4° in a small Lucite cell. The heteroduplex sample was diluted to $A_{260nm} \leqslant 0.03$ for electron microscopy. The remaining procedures for electron microscopy, length measurement, and data computation have been described (6).

Under similar spreading conditions, single-stranded φX174 DNA was 4.6% longer than the double-stranded replicative form and, therefore, lengths of single-stranded regions in heteroduplex molecules were corrected accordingly. The loop positions were determined after the native length of all heteroduplex molecules were normalized to the average length of all molecules in that set.

P2 *vir37* DNA will be referred to as a primary addition mutant [add (*1*)] according to Bellet, Busse, and Baldwin (3), where a segment of wild-type DNA is duplicated once. A secondary addition mutant will have three copies of the same segment in tandem [add (*2*)]. Wild-type DNA by this nomenclature is add (*0*).

RESULTS

P2 vir37/P2 del1 Heteroduplex. The strategy in the following experiments is to form heteroduplexes between an unknown mutant DNA (*vir37*) and a DNA (P2 *del1*) carrying a deletion that has already been mapped. Such a structure should result in two nonhomologous regions and, therefore, the position of the new mutation can be determined.

P2 *vir37*/P2 *del1* heteroduplexes showed two single-stranded loops in otherwise double-stranded molecules. The *del1* loop was easily recognized by its position very close to one end of the molecule [previously determined to be at the right end (6)]. The second loop was, thus, due to *vir37* and was located on the same half as *del1*. The mean position of the loops from the left end of the heteroduplex molecule are shown in Fig. 1*a* and *b*. The *vir37* loop was found to be 0.34 ± 0.03 μm and, within experimental error, corresponds to a unique length (Fig. 1*c*). Our best estimates of the mean position and size of the loops are also shown in Fig. 2.

P2 vir37/P2 vir22 Heteroduplex. The distribution of the *del1* loop position (Fig. 1*b*) is sharp, and the width of the distribution corresponds to the experimental error involved in these measurements. On the other hand, the distribution of the *vir37* loop position (Fig. 1*a*) is somewhat broader than experimental error. This result indicated that the position of the *vir37* loop might be variable. In order to define more precisely the position of the *vir37* addition, we have used an-

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other marker situated closer to *vir37*. The P2 *vir22* deletion occurs between 72.0 and 77.1% from the left end of P2 DNA (6) and should, therefore, be an ideal reference point for the *vir37* loop. Fig. 1*d* shows the observed positions of *vir37* loops measured from the *vir22* reference loop in P2 *vir37*/P2 *vir22* heteroduplexes. The distribution of positions is wider than our experimental error (see legend of Fig. 1*d*) and is thus indicative of a true variation in the *vir37* loop position. The two extreme positions found in the data shown in Fig. 1*d* were 0.04 and 0.35 μm, which represents a range corresponding to 11–95% of the size of the *vir37* loop (in a duplicate experiment where we looked specifically for extreme positions, the limits were 7 and 97%, respectively). In addition, two molecules (<1% of all observations) were found in which both loops had merged into a loop equal to the sum of the *vir37* and *vir22* loops. Thus we conclude that the *vir 37* loop has a variable position that can range from the immediate right of the *vir22* deletion to a rightward position approximating the size of the *vir37* loop. This observation strongly implies that the *vir37* mutation is a tandem genetic duplication (3).

P2 vir37 Homoduplex. In P2 *vir37*/P2 *del1* heteroduplexes, a very low frequency of molecules were seen with only a single loop rather than the two loops discussed above. A careful examination of *vir37*/*vir22* heteroduplexes also showed a single loop (at either the *vir22* or *vir37* position) or two *vir37* loops. These anomalous molecules indicated contamination with DNA from wild type and/or secondary addition mutants in the *vir37* sample and led us to check the homogeneity of the *vir37* DNA. We checked by denaturing and annealing the *vir37* DNA sample alone. Out of 250 molecules scored, looped molecules were again seen and these belonged to three categories: four molecules had a single *vir37* loop (type I), 10 molecules had two loops each of *vir37* size (type II), and three had one loop exactly twice the size of the *vir37* loop (type III) (Plate I). The remaining 233 molecules were completely double stranded. In type-II molecules, the separation between loops varied from 16% to about 108% of the size of the *vir37* loop. Type-III molecules can be produced if the two loops of type-II molecules fuse together. Thus, the extreme separation of the two *vir37* loops varied between 0 and 108% of the *vir37* loop length and indicated that the *vir37* loop could migrate over its entire length. The presence of a few heteroduplex molecules in the annealed DNA solution is consistent with the tandem duplication nature of the *vir37* mutation if there was also present, as a contaminant, both wild-type and secondary addition mutant DNA [add (*0*) and add (*2*)]. Although the type-I heteroduplex could arise by collision between either contaminant [add (*0*) or add (*2*)] and the major species [add (*1*)], the type-II and type-III heteroduplexes should be formed only by collision between the supposed contaminating species [add (0) and add (*2*)]. It is, therefore, surprising that we observe more type-II and -III than type-I molecules. Perhaps, types II and III can arise from collisions between add (*1*) and add (*1*) in which the left duplicated segment of one strand has annealed to the right

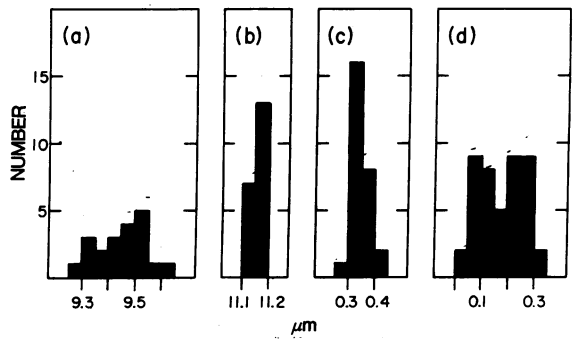

FIG. 1. (*a–b*) Histograms showing the position of the *vir37* addition and the *del1* deletion loops in 20 P2 *vir37*/P2 *del1* heteroduplex molecules. The mean positions were 9.46 and 11.15 μm, respectively, from the left end of the heteroduplex molecules when they were normalized to their average length of 11.2 μm. (*c*) Histogram showing the length of *vir37* loops in *vir37*/*del1* heteroduplexes. Twenty-seven molecules were measured; mean length was 0.34 ± 0.03 μm. (*d*) Histogram showing the native distance between the two loops in *vir37*/*vir22* heteroduplexes. Forty-four molecules were measured; mean length was 0.18 ± 0.08 (±44%) μm. The minimum and maximum separations between the loops were 0.04 and 0.35 μm, respectively, and the *vir37* loop lengths in the two cases were 0.36 and 0.37 μm, respectively. Thus, the separation of the *vir37* loop from the *vir22* loop varied from 11.1 to 94.6% of the *vir37* loop size.

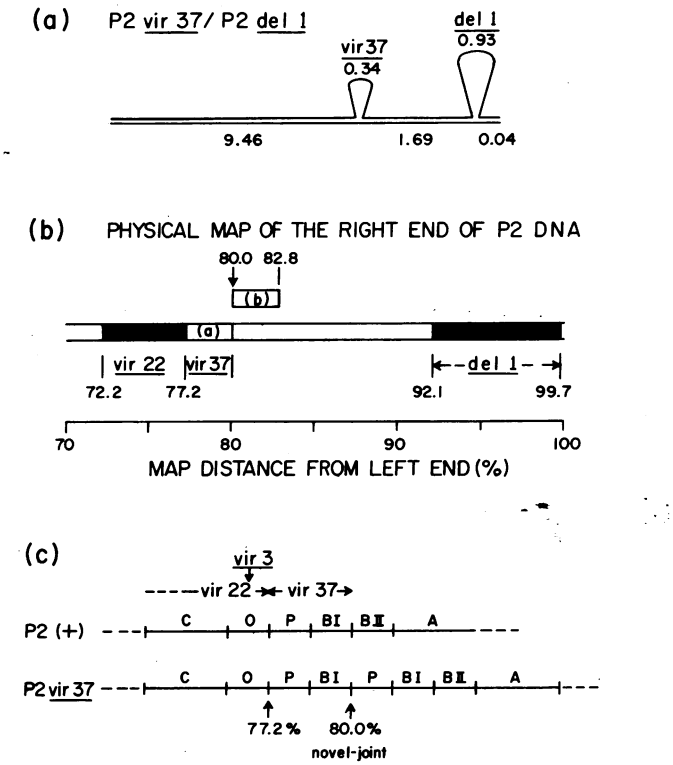

FIG. 2. (*a*) Structure of P2 *vir37*/P2 *del1* heteroduplex molecules (not drawn to scale). The numerical figures represent the length measurements in μm. As discussed in the *text*, the *vir37* loop position was variable. The average position is shown in this figure. (*b*) The position of different mutations in P2 DNA. The position of the *vir22* deletion represents the best estimate from six different heteroduplex experiments done to date (9). The section duplicated in *vir37* (2.8%) is placed to the right of *vir22* [area (*a*)] and the "*novel-joint*" is at 80.0%, as shown by the *arrow* at the top of the figure. At this point another segment identical to sequence (*a*) is present [area (*b*)]. (*c*) Schematic representation of the possible arrangement of genes in the right third of P2 and P2 *vir37*. *C*, repressor; *O*, operator; *P*, promoter; *B* and *A*, "early" genes. *BI* is the segment of gene B that is duplicated in *vir37*, i.e., P and B1 segments are present in duplicate in *vir37*.

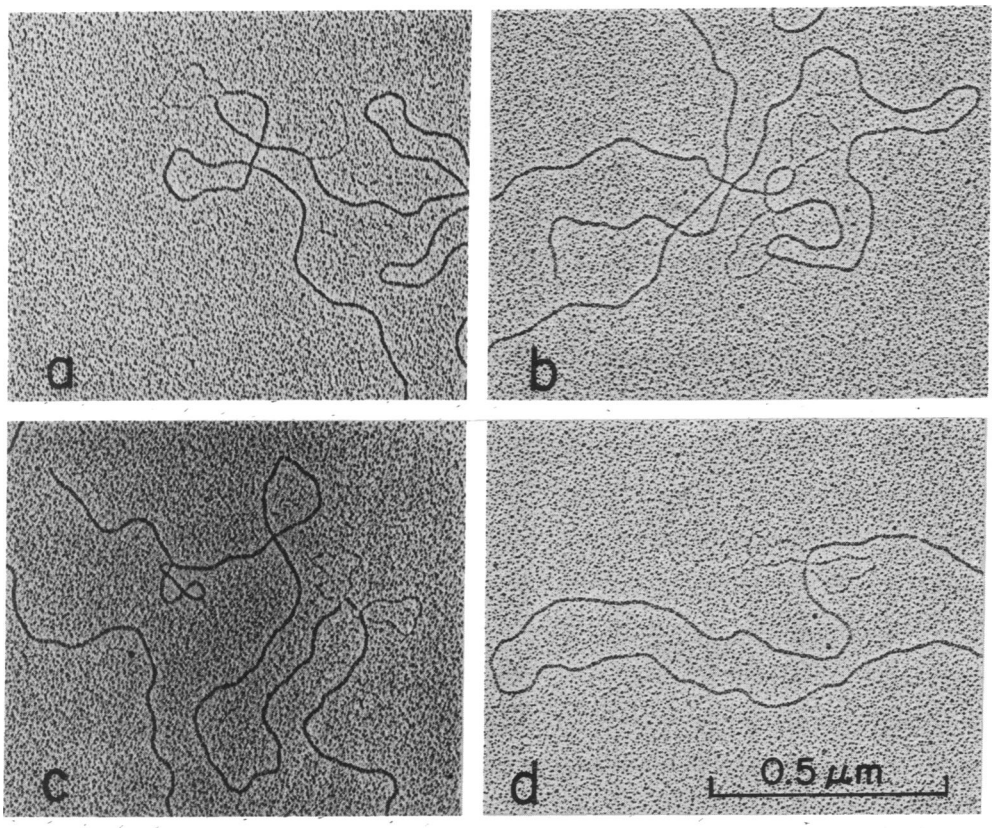

PLATE 1. Electron micrographs showing the heteroduplex loops observed when the P2 *vir37* DNA sample was self annealed. The molecules show two loops each the size of the segment added in *vir37*. The separation between the loops is variable and decreases in the order (*a*) to (*d*). In (*d*), the two loops might have fused to give a single loop twice the size of the *vir37* addition.

duplicated segment of the other strand. In principle, the question can be settled by examination of the detailed structure of the loop in type-III molecules; however, due to the low frequency of this type of heteroduplex (three observations), we cannot at present test and decide between the two possibilities.

Because of the small size of the *vir37* addition, length measurements of native *vir37* DNA could not be used as a test for the presence of add (*0*) and add (*2*) molecules.

DISCUSSION

That *vir37* is an addition mutant was indicated by the increased density of the phage and its heat instability (5), and was confirmed, in the present report, by construction of heteroduplex molecules. The double-stranded length of the P2 *vir37*/P2 *del1* heteroduplex was 11.19 ± 0.21 μm, and the length of the *del1* deletion was 0.93 μm (Fig. 2*a*) [previously found to be 0.94 μm (6)]. Therefore, if the *vir37* mutation is an addition, the P2 wild-type length would be 12.1 μm. Similarly, the double-stranded length of P2 *vir37*/P2 *vir22* heteroduplex was 11.76 μm and the *vir22* deletion was 0.61 μm [previously found to be 0.63 μm (6)]. If allowance is made for the small insertion (0.06 μm) that accompanies *vir22* (6), then the calculated wild-type length is 12.3 μm. Both these lengths agree well with our previous estimate of P2 wild-type length of 12.3 μm under similar spreading conditions (6). The size of the *vir37* loop in P2 *vir37*/P2 *del1* and P2 *vir37*/P2 *vir22* heteroduplexes was 0.34 ± 0.03 μm and 0.35 ± 0.02 μm, respectively. The mean length of *vir37* addition is therefore 2.8% of the P2 chromosome. Density measurements on the phage also indicated a 2.8% addition (5).

We have shown that *vir37* exhibits the properties expected of a duplication mutant (4). The location of the *vir37* addition on the P2 chromosome is imprecise because of the variable position of the *vir37* loop in the heteroduplex molecules; however, we have shown that the *vir37* loop can migrate as far to the left as the position of the *vir22* deletion (the two loops can, in fact, sometimes fuse together in a P2 *vir22*/P2 *vir37* heteroduplex). This guides us to place the *vir37* DNA segment to the immediate right of the deleted segment in *vir22*. Whether there is a small degree of overlap or separation between the *vir37* segment and the *vir22* deleted sequence cannot be determined from the present data. If there is no overlap between the deleted and added segments, then the *vir37* loop in P2 *vir37*/P2 *vir22* heteroduplexes should migrate over a distance equal to the size of the *vir37* loop. Partial overlap, on the other hand, will decrease the distance over which the *vir37* loop will migrate. We observe separations between *vir22* and *vir37* up to 97% of *vir37* loop length and, therefore, conclude that most likely there is little, if any, overlap or separation and that the base sequence duplicated in *vir37* starts to the immediate right of the sequence deleted in *vir22*. Taking the *vir37* primary addition to be 2.8% of the P2 chromosome, the position of *vir37* therefore extends from 77.2 to 80.0% from the left end of P2 DNA [the right end of the *vir22* deletion terminates 77.2% from the left end (Fig. 2b)]. As the *vir37* segment is present in duplicate, the "novel-joint" (8) should be 80.0% from the left end of P2 DNA. To

the right of this position is an identical base sequence extending to 82.8%, as shown in Fig. 2*b*. The "novel-joint" in the case of *t del33* DNA, a derivative of λ phage, was determined with high precision from different overlapping duplication mutants (4). Such an approach was not possible in the present study since other P2 duplication mutants were not available.

P2 *vir37* has shown many of the physical properties expected of a duplication mutant; however, additions higher than add (*1*) have not so far been detected (5). Primary additions in the case of *vir37* were obtained from wild-type P2 rather than from a deletion mutant, as in the case of *t del33* which is 0.77 of the length of λ (4). This is significant since the frequency of occurrence of add mutants in *t del33* showed a dependence on the length of DNA addition. Between the interval 0.91 and 1.02 of the length of λ (after addition), the frequency of addition mutants did not change but dropped by more than an order of magnitude as the length increased from 1.02 λ to 1.10λ (Baldwin, personal communication). The P2 *vir37* primary addition mutant is 1.03 P2 length; hence, the chance of survival of higher order additions is less and may explain why they have so far not been detected. The small contamination of wild type (and possibly a secondary addition mutant), described in *Results*, in the *vir37* stock sample is consistent with the segregation pattern of a primary duplication mutant.

In a previous study, a tentative relationship between the physical and genetic maps of P2 was drawn with respect to the *vir22* position in the two maps (6). The present study shows that *vir37* is located to the right of *vir22;* this should therefore also be the order on the genetic map. Two other mutants of P2, *vir22* (6) and *vir79*, have been physically mapped, and like *vir37*, give rise to an immunity insensitive phenotype (G. Bertani, unpublished observation). Both these are deletion mutants, and the region deleted in *vir79* is nested within the *vir22* segment (D. K. Chattoraj and R. B. Inman, unpublished observation). The region deleted in *vir22* occurs 72.2–77.2% from the left end of P2 DNA (9). According to our best estimates, this region is unaffected in *vir37* and the first chromosomal aberration starts 80% from left end (at the "novel-joint") and is separated from *vir22* by 2.8% of P2 DNA. Genetic analysis shows that the *vir37* addition includes part of gene B (5); therefore, the "novel-joint" is situated in gene B and all genes to the left of B are intact in *vir37*. The known genes to the immediate left of gene B include the repressor gene C and the binding site of repressor protein characterized by the *vir3* mutation (10); hence, these genes should be intact in *vir37*. Since, in *vir37*, the B, A genes are transcribed constitutively, these genes must be present intact, at least in one copy, and the promotor site for B, A transcription must somehow be independent of the repressor-operator action. Fig. 2*c* shows one possible genetic sequence that accounts for many of the above findings. A new copy of the existing promoter is created by duplication and is separated from the operator. The expected position of *vir22* deletion is also shown, since we find that the *vir37* addition is just to the right of the *vir22* deletion. The second fact bearing on this model comes from genetic experiments showing that the operator region is nonfunctional and most probably deleted in *vir22* (5). Though alternative possibilities are not excluded, this model is consistent with much of the genetic and physical mapping data on the immunity insensitive mutants of P2. A more complete consideration of different possible gene arrangements in P2 *vir37* is described in the adjoining paper (5).

Another P2 mutant, P2 *sig5*, has been characterized by density measurement to be an addition (about 2% of the size of P2 DNA), and its position on the P2 genetic map is between genes C and B (11). It appears that *vir37* and *sig5* are located in the same region of the genetic map and both are addition mutants. A mechanism analogous to that shown in Fig. 2*c* might explain why P2 *sig5*, as a prophage, can form the *cis*-acting protein coded by gene A of P2 (11).

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