*ELECTRON-MICROSCOPIC VISUALIZATION OF DELETION MUTATIONS**

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Communicated February 12, 1968

Deletion mutants of λ coliphage were discovered by Kellenberger, Zichichi, and Weigle.[1] The deletions can be mapped by genetic recombination experiments. It is not known if the recombination maps thus obtained give the true physical position of the deletions. The present paper describes a method for mapping deletion mutations with the electron microscope, thus obtaining the physical position of the deletion. The basic principle is as follows. A mixture of DNA molecules from wild-type virus and from the deletion mutant is subjected to strand dissociation followed by reannealing conditions. The resulting preparation contains some double-stranded renatured molecules of each type and some heteroduplexes. In the latter, one strand is wild-type DNA and the other is deletion mutant-type DNA. Each heteroduplex should thus contain a single-stranded loop in the wild-type DNA strand at the point where the deletion occurs. The contour lengths of double-stranded regions of a DNA molecule can be rather accurately measured in electron micrographs. Under the conditions used to prepare the electron microscope grids, the single-stranded loops are collapsed into "bushes" similar to those observed in T_2 phage DNA by MacHattie, Ritchie, Thomas, and Richardson.[2] The contour length of such a bush can only be roughly estimated at best, but the bush position with respect to the double-stranded regions can be accurately measured.

Materials and Methods.—The c_I point mutant λc_{26} and the deletion mutants $\lambda b_2b_5c_1$, λb_5c_2 (Kellenberger, Zichichi, and Weigle[1]), and $\lambda b_{221}c_{26}$ (Huskey[3]) were obtained from Dr. Robert Huskey. Phage were grown and purified by standard methods, including banding in CsCl by density gradient centrifugation.[4] The phage solution was finally dialyzed against 0.01 *M* $MgSO_4$, 0.01 *M* tris buffer (pH 7).

A typical heteroduplex preparation was made as follows. Ten μl of 0.1 *M* ethylenediaminetetraacetate (EDTA) (pH 8.2), 15 μl of 5 *M* NaCl, 46.6 μl of H_2O, 5.1 μl of λc_{26} phage solution (A_{260} = 10.0), 3.4 μl of λb_5c_2 solution (A_{260} = 14.2), and 10 μl of 1 *N* NaOH were added, in the order given, to a small test tube (6 × 50 mm). The mixture (pH 13) was incubated at room temperature for 10 min and then chilled in an ice bath. The alkaline solution causes lysis of the phage and dissociation of the DNA into single strands. The solution was neutralized by addition of 10 μl of 2 *M* NaH_2PO_4 (final volume, 100 μl). The DNA was partially renatured by heating the solution to 70°C for 30 sec and then quickly cooled (these conditions were selected to give over 50% renaturation, minimal single-strand scissions, and minimal higher-order aggregation[5]). All other preparations were done similarly except that the volume of phage suspension added was varied to give equal numbers of the two phage types with a combined A_{260} = 1.0 (*ca.* 10^{11} total phage). In control experiments only one type of phage DNA was present.

Grids for electron microscopy were prepared by the basic protein film technique[6] and stained with uranyl salts.[7] A solution containing 0.5 μg/ml of DNA and 0.1 mg/ml of cytochrome *c*, in 0.5 *M* ammonium acetate, 0.001 *M* EDTA (pH 7.9), was spread onto 0.15 *M* ammonium acetate (pH 6.5). The cytochrome *c*–DNA-mixed film was picked up on grids freshly covered (less than 2-days old) with films prepared from 3% w/v

Parlodion in amyl acetate. Best staining results were obtained when the droplet of solution that was picked up with the cytochrome *c*–DNA film gave a flat meniscus on the Parlodion grid. The grids were stained by dipping them for 30 sec in a 90% ethanol solution of stain and then rinsed for 10 sec in isopentane. The best stain solution was a 1000-fold dilution into 90% ethanol of a stock solution of either 0.05 *M* uranyl chloride (made from uranyl oxide) in 0.05 *N* HCl or 0.05 *M* uranyl acetate in 0.05 *N* HCl. The stock solutions were stored in the dark. A fresh, diluted stain solution was prepared for each set of grids and was used within 1 hr after dilution. Some grids were also shadowed with platinum-palladium. DNA molecules were photographed with a Phillips EM 200 electron microscope using a 50-μ objective aperture and 60-kv accelerating voltage. The resulting negatives (approx. 3600X) were enlarged ten times and traced on a Nikon shadowgraph. Lengths were measured on the tracings with a map measurer. Fifteen to 40 molecules were measured for each heteroduplex and each control. Bush sizes were estimated by enlarging (50X) the micrographs on the shadowgraph and tracing the periphery of the bush.

The size of a deletion was determined with the electron microscope by a direct comparison of the length of wild-type and deletion mutant DNA's on the same grid. This procedure virtually eliminates magnification errors and differential environmental effects on the length of DNA. Phenol-extracted native DNA was used for each determination One comparison was also made using heat-shocked phage (300-fold dilution of phage stock into 0.001 *M* EDTA, 10 min at 37°C). Since the b_5 deletion is small, the histograms of λb_5c_2 and λc_{26} might overlap. Therefore, in order to identify the type of DNA in the electron microscope, circular (along with some linear) λc_{26} DNA was compared to linear λb_5c_2 DNA, and circular (along with some linear) λb_5c_2 DNA was compared to linear λc_{26} DNA. Grids were stained with uranyl chloride and shadowed with platinum-palladium (to prevent the Parlodion film from stretching).

Results and Discussion.—Ideally, when a mixture of two DNA's, one of them differing from the other by a genetic deletion, is denatured by alkali, neutralized, and heat-annealed, a mixture containing each of the two original double-stranded DNA's and a heteroduplex double-stranded DNA is obtained. This heteroduplex has, at the point of the deletion, a single-stranded bush which can be readily identified by electron microscopy.

Before presenting the results, certain difficulties in interpretation due to the formation of molecules with bushes that are not due to a deletion should be mentioned. These cases are probably due to imperfect annealing and/or to the occurrence of single-strand breaks. Some completely denatured single-strand molecules are seen but are readily identified (Fig. 2*a*, arrow). In a typical preparation approximately 33 per cent of the renatured molecules showed bushes on one or both ends. We believe that this end-bush formation results from the renaturation of two strands, of which one or both is incomplete due to a prior single-strand break. The double-stranded portion of such a molecule was always found to be shorter than a whole double-stranded molecule. For a duplex containing one unbroken and one broken strand, the estimated length of the bush (or bushes) should increase linearly with decreasing length of the double-stranded region. The correlation between measured periphery lengths of end bushes and lengths of the double-stranded regions is displayed in Figure 1. The data give the predicted linear relation and a conversion factor of 1/3.2 between bush periphery length and double-stranded length. The single-strand breaks were mapped (by the length of the double-stranded region) and appear to be randomly located.

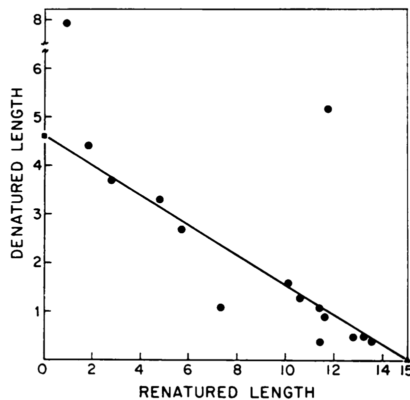

FIG. 1.—The lengths of the double-stranded portions of renatured molecules with end bushes are plotted against the lengths of the peripheries of the end bushes. The straight line is drawn between whole single-stranded DNA (■) (mean of eight measurements) and whole double-stranded DNA (▲) (mean of 31 measurements). It is seen that the ratio of the double-stranded contour length of a whole molecule to the single-stranded periphery length of a whole strand is 3.2. The two points far above the line are probably the result of aggregation of a whole single strand with an end bush. The two points below the line are probably the result of annealing two fragmented single strands.

Considering now only the renatured molecules without end bushes, in all heteroduplex preparations approximately half the molecules showed internal bushes, while in control preparations less than 5 per cent of the molecules showed internal bushes. The internal bushes on renatured molecules in the control preparations (that is, from a single kind of DNA) are probably mainly due to nonspecific aggregation of a single-stranded segment with a renatured two-stranded molecule or to hybridization of, for example, a broken *W* strand with a whole *C* strand, followed by renaturation with an additional whole *W* strand. The internal bushes in the control preparations appear to be randomly located. As noted below, the internal bushes that are identified as a deletion always occur at a unique location.

In heteroduplex preparations, pictures were taken of all isolated molecules which showed internal bushes and which were sufficiently straightened out so that intramolecular overlaps caused no ambiguity as to the position of the bush. Typical molecules are shown in Figure 2.

The measured positions of the bushes in the heteroduplexes can be used to calculate the positions of the deletions in the wild-type DNA if the size of the deleted region is independently known. This calculation proceeds as follows. Let L_0 be the physical length of the wild-type molecule, with X_1^i and X_2^i the physical positions (measured from the left end) of the beginning and end of the *i*th region, which is missing in the deletion mutant, *i*. The physical length of the deletion is $D^i = X_2^i - X_1^i$. The fractional or map positions are $x_1^i = X_1^i/L_0$ and $x_2^i = X_2^i/L_0$, and the fractional size of the deletion is $d^i = D^i/L_0 = x_2^i - x_1^i$. The deletion-mutant molecule has a length $L_0 - D^i$; the total length of the double-stranded regions of a heteroduplex between a wild-type and an *i*-type molecule is also $L_0 - D^i$. Therefore, the physical distance from the left end of the heteroduplex to the bush due to region *i* is X_1^i; the fractional length is $X_1^i/(L_0 - D^i)$ or $x_1^i/(1 - d^i)$. In a heteroduplex between a molecule with deletions of the two nonoverlapping regions *i* and *j* and a molecule from which only *i* is missing, the bush due to the *i*th region occurs at $x_1^i/(1 - d^i - d^j)$ or at $(x_1^i - d^j)/(1 - d^i - d^j)$, depending on whether the *i*th region is to the left or the right of the *j*th region.

In a study of the molecules from the b_5 heteroduplex preparation ($\lambda b_5c_2 \times \lambda c_{26}$) (Fig. 2*c*), the internal bushes were found to be uniquely located as shown by

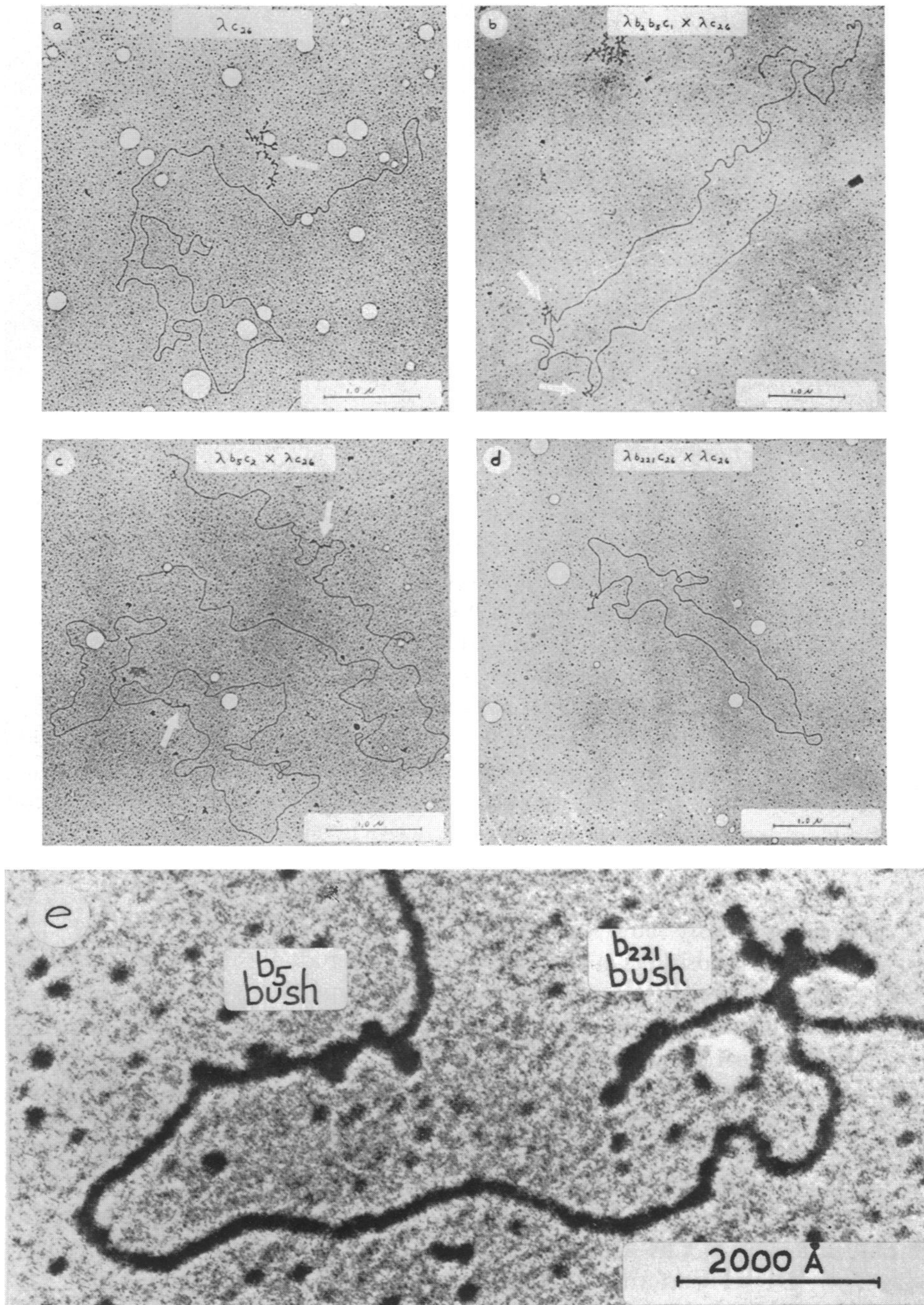

FIG. 2.—Electron micrographs of renatured λ DNA, stained with uranyl salts.

(a) λc_{26} DNA self-annealed. Arrow shows a single strand of DNA.

(b) $\lambda b_2 b_5 c_1 \times \lambda c_{26}$ heteroduplex showing two bushes (*arrows*).

(c) Two $\lambda b_5 c_2 \times \lambda c_{26}$ heteroduplexes, a circular and a linear molecule, each showing one small bush (*arrows*).

(d) $\lambda b_{221} c_{26} \times \lambda c_{26}$ heteroduplex showing one large bush and showing an apparently pulled-apart circle.

(e) Enlarged micrograph showing the b_5 and b_{221} bushes.

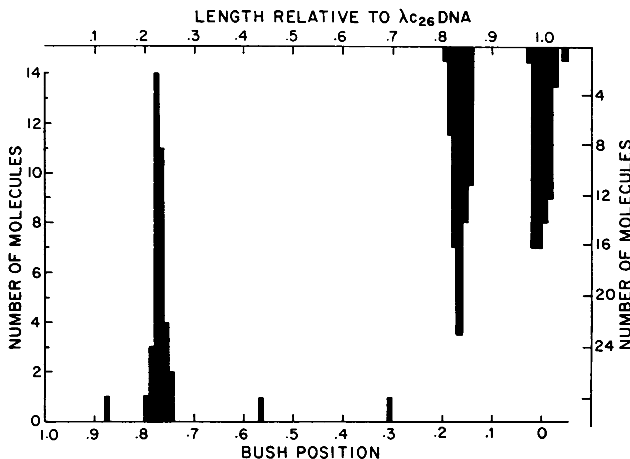

FIG. 3.—Histogram of bush positions for molecules with internal bushes in a $\lambda c_{26} \times \lambda b_5 c_2$ heteroduplex preparation. The horizontal coordinate is the distance of a bush from an end, measured as a fraction of the renatured molecular length, and is therefore the quantity $x_1^{b5}/(1 - d^{b5})$.

Histogram of the relative lengths for a mixture of λc_{26} and $\lambda b_2 b_5 c_1$ DNA molecules on the same grid. The mean of the λc_{26} DNA lengths is taken as 1.00. The standard deviation of the length measurements is 1.2%.

the histogram in Figure 3. Ignoring the three randomly located bushes, we find the mean position of the other 35 to be $x_1^{b5}/(1 - d^{b5}) = 0.770$ with a standard deviation of $\pm$ 0.009. Since the b_5 deletion alters the product of the c_I gene, one might assume that this deletion involves or is near the c_I gene.[8] The c_I gene genetically maps in the right quarter of the λ DNA molecule, and this is in good agreement with the bush position if the left end is taken as 0 and the right end as 1 unit of length.

A better deletion to study is the b_2 deletion, since there is good genetic evidence that this deletion is in the center of the λ DNA molecule.[9] However, it is not known if this deletion is slightly left or right of center; therefore, the double deletion b_2 and b_5 heteroduplex was prepared ($\lambda b_2 b_5 c_1 \times \lambda c_{26}$). As expected, this heteroduplex shows two internal bushes (Fig. 2*b*). These internal bushes are uniquely located at 0.736- and 0.552-molecular lengths (one molecular length now being that of the double deletion $b_2 b_5$ heteroduplex DNA molecule). If these two bushes are the b_5 and b_2 regions, respectively, then $(x_1^{b5} - d^{b2})/(1 - d^{b5} - d^{b2}) = 0.736$ and $x_1^{b2}/(1 - d^{b5} - d^{b2}) = 0.552$.

To show that the bush at 0.552 molecular length is the b_2 bush, the $\lambda b_2 b_5 c_1 \times \lambda b_5 c_2$ heteroduplex was prepared. This heteroduplex should show only the b_2 bush, since both DNA strands have the b_5 deletion. A single bush is observed which maps at $x_1^{b2}/(1 - d^{b5} - d^{b2}) = 0.544$.

If we take $d^{b2} = 0.112$ and $d^{b5} = 0.053$ (see below), the observation that $x_1^{b5}/(1 - d^{b5}) = 0.770$ implies that $x_1^{b5} = 0.729$. This predicts that $(x_1^{b5} - d^{b2})/(1 - d^{b2} - d^{b5}) = 0.738$, in satisfactory agreement with the observed value, 0.736. The position of the b_2 deletion is calculated as $x_1^{b2} = 0.458$. Then $x_2^{b2} = 0.570$; and the b_2 region is seen to be very close to the center of the molecule.

To compare further genetic and DNA distance mapping, a new, large deletion mutation, b_{221}, isolated by Dr. Robert Huskey, was studied. His genetic studies[3] show that this deletion overlaps the b_2 deletion and extends farther on both sides of it. The b_{221} heteroduplex ($\lambda b_{221} c_{26} \times \lambda c_{26}$) gives a single, large internal

TABLE 1. *Length and bush position for renatured molecules, and deletion region coordinates.*

Renatured DNA	Length in μ	Bush position in fractions of the molecular length	Deletions Regions in Fractions of the Wild-Type DNA Length Measured from Left End	
λc_{26}	15.0 ± 0.4			
λb_5c_2	13.0			
$\lambda b_5c_2/\lambda c_{26}$	13.3	0.770	$x_1^{b5} = 0.729$	$x_2^{b5} = 0.782$
$\lambda b_2b_5c_1$	11.7			
$\lambda b_2b_5c_1/\lambda c_{26}$	11.6	0.552	$x_1^{b2} = 0.461$	$x_2^{b2} = 0.573$
		0.736	$x_1^{b5} = 0.727$	$x_2^{b5} = 0.780$
$\lambda b_2b_5c_1/\lambda b_5c_2$	11.7	0.544	$x_1^{b2} = 0.454$	$x_2^{b2} = 0.566$
$\lambda b_{221}c_{26}$	11.2			
$\lambda b_{221}c_{26}/\lambda c_{26}$	11.2	0.523	$x_1^{b221} = 0.406$	$x_2^{b221} = 0.629$
$\lambda b_{221}c_{26}/\lambda b_5c_2$	9.8	0.568	$x_1^{b221} = 0.411$	$x_2^{b221} = 0.634$
		0.703	$x_1^{b5} = 0.732$	$x_2^{b5} = 0.785$

bush at 0.523-molecular length from an end (Fig. 2*d*). In order to locate this deletion, left or right of center, the double-deletion heteroduplex ($\lambda b_{221}c_{26} \times \lambda b_5c_2$) was studied. This heteroduplex gives two internal bushes (Fig. 2*e*), one mapping at 0.703 and the other at 0.568-molecular lengths. Calculation then shows that the b_{221} deletion is centered in the right half of the λ DNA molecule, with $x_1 = 0.408$ and $x_2 = 0.631$. Thus, this deletion overlaps the b_2 deletion, in agreement with genetic mapping.

One further control is that the deletion mutant strand of DNA in a heteroduplex should define the length of the heteroduplex DNA molecule so that the heteroduplex molecule should have the same length as the self-annealed deletion mutant DNA molecule. That this is the case is demonstrated in Table 1. It should be pointed out that good estimates of the per cent deletion cannot be made from these length measurements, since the conditions which cause fluctuations in length were not rigidly controlled.[10] Good estimates of the per cent deletion were obtained, however, by mounting both deletion mutant and wild-type DNA's (phenol-extracted native DNA) on the same grid. As shown in Figure 3, sharp symmetrical histograms were obtained with a standard deviation of about 1 per cent. The per cent deletion was also determined, using the buoyant densities of the whole phage.[11] All these data are presented in Table 2. It is seen that the per cent deletion calculated from buoyant density agrees

TABLE 2. *Per cent deletion by electron microscopy and by buoyant density.*

DNA	Form	Per cent deletion elec. micro.*	Method of preparation	No. measured	Buoyant density of phage†	Per cent deletion by buoyant density‡
λb_5c_2	Linear	5.3	Phenol-extracted	54	1.502	5.5
λb_5c_2	Circular	5.2	Phenol-extracted	59	—	—
$\lambda b_2b_5c_1$	Linear	16.5	Phenol-extracted	99	1.487	17.9
$\lambda b_2b_5c_1$	Linear	16.5	Heat-shocked	36	—	—
$\lambda b_{221}c_{26}$	Linear	22.3	Phenol-extracted	82	1.480	23.1

* Per cent shorter than λc_{26} linear (or circular) DNA.

† Buoyant density in CsCl of $\rho^{20} = 1.493$, taking the buoyant density of the λc_{26} marker as 1.508.

‡ Per cent deletion $= -100\Delta\rho/(0.106 - 0.54\ \Delta\rho)$. This equation follows from equation (1) of Weigle *et al.*,[11] by taking $\rho_{DNA} = 1.704$ and $\rho_{protein} = 1.28$. The quantity $\Delta\rho$ is the buoyant density of the mutant phage minus that of λc_{26} phage and is negative, corresponding to a positive value of the per cent deletion.

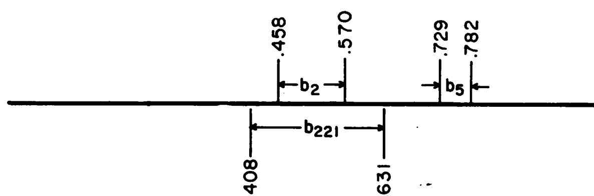

FIG. 4.—Summary of results for positions of the b_{221}, b_2, and b_5 regions.

rather well with that measured by electron microscopy. We estimate the standard deviation in our determination of deletion size as ±0.02-molecular lengths. In general, these measured deletion sizes are slightly smaller than those reported in a number of other investigations by several physical methods, but the discrepancies appear to be within the estimated respective errors. The values for x_1 and x_2 for the several deletions studied, as quoted above, were calculated using the values for the fractional deletion size (*d*) measured by electron microscopy (Table 2). These results are summarized in Table 1 and Figure 4.

The results of the present physical mapping study can be compared with results obtained in several other studies. Skalka, Burgi, and Hershey[12] have deduced the positions of segments of differing base composition along the λ DNA molecule by a physical method. They conclude that there is a segment of low GC mole fraction (0.37) extending from 0.44 to 0.54 of the λ DNA molecule and largely missing in λb_2 mutants. The position of the b_2 region, as determined by us, is from 0.455 to 0.567. These results support the inference that the b_2 region and the segment of low GC content are virtually identical. The position of the *N* gene has been determined by a method based on the infectivity of fragments of different sedimentation coefficients to be 0.27 molecular lengths from the right end of the λ DNA molecule.[13] The *N* gene is close to the left end of the b_5 region, which we map at 0.273 lengths from the right end of the molecule.

Genetic recombination frequencies between the boundaries of the b_2 region and various markers to the left and the right have been studied by several investigators. Similarly, mutations in the c_I gene, which is in the b_5 region, have been genetically mapped.[3, 8, 9, 14–16] All of these results are in semiquantitative agreement, at least, with the physical data presented here. A quantitative critical collation of the genetic recombination data is beyond the scope of the present contribution.

In the course of these experiments several interesting incidental observations have been made. All preparations contained some hydrogen-bonded circular molecules. In several cases, ends which were close and which appear to have been pulled apart during or after incorporation in the cytochrome film were observed in both heteroduplex and self-annealed molecules (Fig. 2*d*). The "break" always occurred at the cohesive site as determined by bush position. The two ends are not connected by single-stranded DNA, for single-stranded DNA can be seen by the grid preparation technique used.

Another observation concerns the appearance of the b_5 bush. The bush formed by a single continuous deletion should originate from a single point. This is true for all the b_2 and b_{221} bushes that were observed. However, the b_5 bush

does not appear at one point; rather, it is usually spread over a region (the longest regions were 0.25-μ long) with up to five small "bushlets." These phenomena are illustrated in Figure 2*e*. Out of 122 well-stained b_5 bushes studied at high magnification, only eight appeared as a single bush, while all others appeared as multiple bushlets. Attempts were made to map the multiple bushlets, but no unique positions could be found. These characteristics of the b_5 bush indicate that the b_5 deletion is not a simple continuous deletion. In view of the altered immunity caused by the b_5 mutation,[8] it would appear that the b_5 deletion is really a DNA substitution, in which the substituted piece contains 5.3 per cent less DNA and the substitution shows partial homology with wild-type λ DNA.

It is anticipated that the method described here will be useful for a number of problems involving nucleic acid homology.

Summary.—Heteroduplex DNA molecules in which one strand is from the wild-type viral DNA and the other strand from the DNA of a deletion mutant strain can be prepared by renaturation. These molecules consist mainly of double-stranded regions and are readily visualizable in the electron microscope by the basic protein film method. They show a bush (collapsed single-stranded loop) at the point where the deletion occurs. The bush positions can be accurately measured. The size of the deletion can be measured from the difference in contour length of the native wild-type DNA molecules and native deletion mutant DNA molecules. These two measurements permit a physical mapping of the position of the deletion regions. The b_2, b_5, and b_{221} deletions in λ DNA have been so mapped and the final results are presented in Figure 4.

After completing this manuscript we have learned that the technique described herein has been independently conceived and developed by Westmoreland, Szybalski, and Ris, who have obtained results which are generally similar to ours.

We are grateful to Dr. Robert Huskey for his help and phage stocks, to Dr. James G. Wetmur for advice on annealing conditions, and to Dr. Jean Weigle for his patient guidance and counsel in many matters. This work has been supported by research grant GM 10991 and training grant GM 1262 from the U.S. Public Health Service.

* Contribution no. 3649.

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