The Structure and Function of the DNA from Bacteriophage Lambda

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ABSTRACT The position and orientation of genes in lambda and lambda dg DNA are described. The position of six genes located in the right half of isolated lambda DNA was found to be -(N, i^{λ})--*O*-*P*---*Q*-*R*-(right end of DNA), which is their order on the genetic map of the vegetative phage. The order of the three genes of the galactose operon (*k*, *t*, and *e*) located in the left half of lambda dg DNA was found to be (left end of DNA)----*k*-*t*-*e*-, consistent with Campbell's model (5) for the formation of this variant. Gene orientation, defined as the direction of transcription along the DNA, is inferred to be from right to left for the galactose operon in lambda dg DNA. The strand of lambda DNA which functions as template in transcription of *N*, an "early" gene required for normal replication of lambda DNA, was determined as a first step in ascertaining the orientation of this gene. The method includes isolation of each strand, formation of each of two heteroduplex molecules consisting of one strand from wild-type and one from an *N* mutant, and comparison of their *N* activities. The second step, which consists of ascertaining the 5′-to-3′ direction of each strand, is discussed, as is a determination of the orientation of gene *R*.

INTRODUCTION

The Genetic Maps of Lambda

In considering the structure and function of the DNA from bacteriophage lambda, it is convenient to make use of the order of the genes on the genetic map as the point of departure and as a standard to which the DNA can be compared. Bacteriophage lambda is episomic and consequently its genome exists in at least two states within which genetic recombination is possible. This allows the construction of two genetic maps that are termed "vegetative" and "prophage" after these states. In the vegetative state, the replication of the lambda genome is independent of the replication of the host (*Escherichia coli*) genome. Such replicas are finally packaged into the head of the mature phage as single duplex DNA molecules, 15 to 17 microns in length (1–4). These molecules contain some 47,000 base pairs, enough for 40 to 45 structural genes, each capable of specifying a polypeptide of 40,000 molecular weight.

In the prophage state the lambda genome is thought to exist as an integral part of the DNA of the resulting lysogenic bacteria. A consequence of this insertion is the replication of the lambda genome as a set of contiguous genes within the bacterial chromosome, doubling with each cell generation.

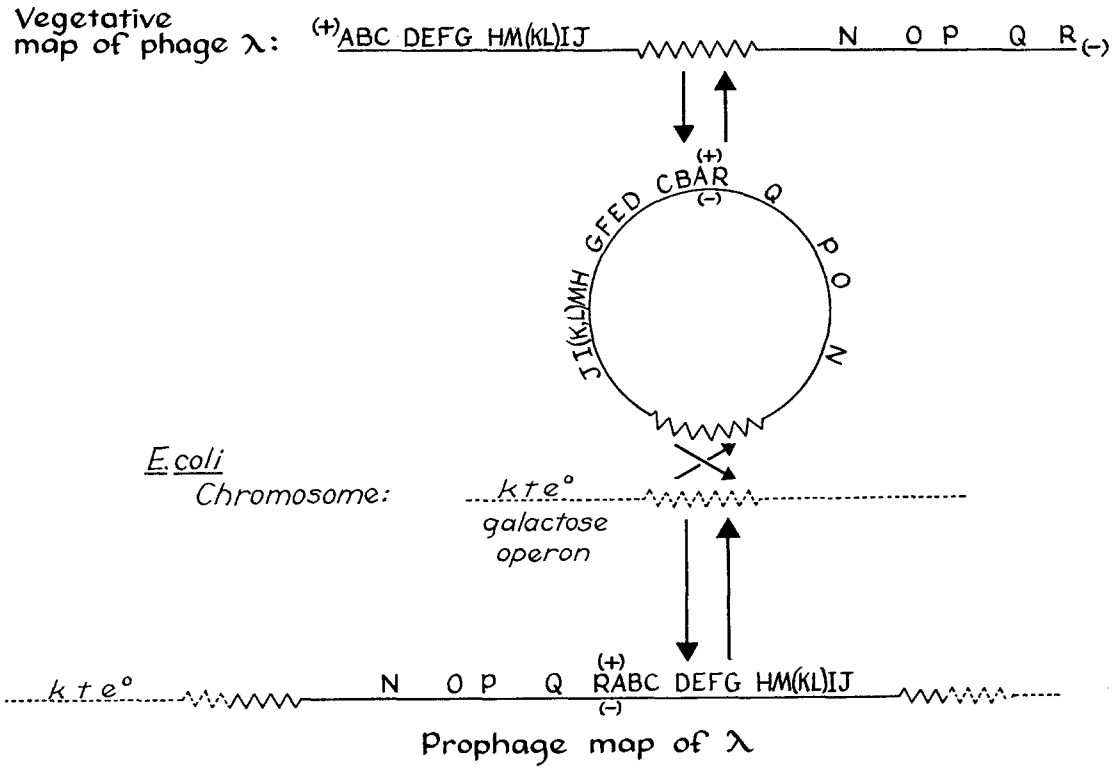

FIGURE 1. The Campbell (5) relation between the vegetative and prophage maps. The capital letters *A* through *R* represent lambda genes. The lower case letters *k*, *t*, and *e* represent three genes of the galactose operon which determine the structure of the enzymes galacto*kinase*, galactose-1-P uridyl *transferase* and, UDP-galactose 4-*epimerase*, respectively (14–16). The *o* associated with the galactose operon designates the end containing the operator (16).

The two genetic maps constructed from these two states of the lambda genome, though different, are related by a circular permutation. This is shown in Fig. 1 which depicts Campbell's (5) model for relating the two maps. The genes, *A* through *R*, are ordered on the vegetative map as a linear array bounded by two ends, (+) and (−). We need not define the idiosyncrasies of these 18 genes now; rather, consider them as position markers on the map or in the DNA. The order shown in the vegetative map is well defined, though the position of the genes is only approximate (4, 6–11).

In the model the ends [(+) and (−)] of the infecting DNA join together to make the closed form shown in the center of the figure. The prophage map can be generated from the closed form by breaking it opposite the (+), (−) joint, somewhere in the region of the wavy line. Campbell (5) suggested that

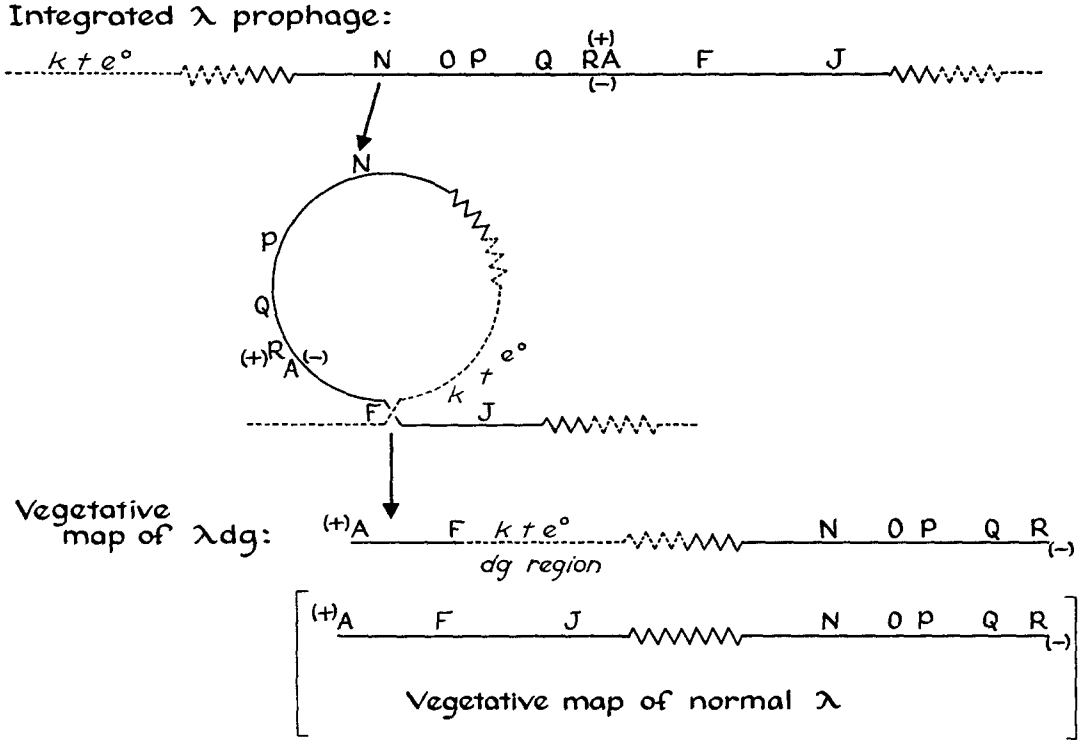

FIGURE 2. Formation of lambda dg from prophage. The symbols are those defined in Fig. 1. Independent isolates of lambda dg are different in respect to the left-hand end point of the dg region, which is defined by both the extent of the deficiency in lambda genes and the extent of addition of *E. coli* genes. Starting at and always including the *K, L, I, J* group, the deficiency can extend to the left to a varying extent, in some cases including the entire *A*-to-*M* group (see Fig. 1) (7). The amount of the galactose operon added in lambda dg can also vary, the excluded portion always extending from the left-hand end of *k* to the right into *t* (18); however, most lambda dg contain the entire operon. To comply with these facts the model must allow for variation in the position of the crossover that will yield closed forms of differing size.

this break and the subsequent integration into the host chromosome took place by a reciprocal recombination between this region in the lambda circle and an homologous region in the host DNA located close to the site of the galactose operon. This aspect of lysogenization might be referred to as a "Campbellism" of lambda by *E. coli.*

If the recombination were to take place in the direction of the arrows, then

the order of phage and bacterial genes would be as shown in the lower line. This order is consistent with the prophage maps that have been constructed (11–13).

These then are the two gene orders of normal lambda that have been established by genetic mapping procedures. They relate to two aspects of lambda DNA which will be considered here. The first concerns the position of these genes in the lambda DNA molecule as isolated from mature phage. The second concerns the orientation of each gene. Gene orientation will be defined more explicitly later; for the present, consider it as synonymous with the direction of transcription along the DNA.

In order to include bacterial genes which are closely linked to prophage lambda within these considerations, the DNA from the lambda dg variant will also be discussed. The relation that lambda dg bears to normal lambda can be more easily understood by referring to Fig. 2 which depicts Campbell's (5) model for the formation of lambda dg from prophage.

It is supposed that when a lysogenic bacterium is induced to yield phage that the reciprocal recombination necessary to pop-out the closed form does not, in the formation of lambda dg, take place between the regions of homology used in the formation of normal lambda. Rather, it is supposed that very rarely such a recombination occurs at some region of minor, accidental homology such that part of the *E. coli* chromosome is included in the loop—specifically, the galactose operon. When the closed form that results from the recombination is opened at the (+), (−) joint, the vegetative map of lambda dg is obtained. This contains a region—called the dg region—which is deficient in lambda genes, containing in their stead the galactose operon and perhaps other unidentified genes of *E. coli*. The position of the defective aspect of this region relative to the vegetative map of lambda has been determined by Arber (17) and by Campbell (6, 7) to be that shown in Fig. 2. However, the orientation and position of the galactose operon on the map of lambda dg have not been directly determined.

THE ORDER AND POSITION OF GENES IN LAMBDA AND LAMBDA dg DNA

With the genetic maps of lambda and lambda dg as guides, consider now the actual disposition of genes in the DNA isolated from these phages. The isolated lambda DNA can exist in open or closed monomeric forms which exhibit the same contour lengths in the electron microscope and are interconvertible (1, 2, 4, 19). The dependence of the stability of each molecular form on salt concentration and temperature (4, 19) suggests that the cohesive sites at each end of the open form consist of a small number of unpaired bases resulting from the protrusion of one strand over the other; the closed form would result from the pairing of the bases in one protrusion with those in the

other. This model is consistent with the complementary relation between the cohesive sites (20), their sensitivity to DNA polymerase and exonuclease (21), and the formation of end-to-end aggregates of the lambda DNA monomer (1, 19).

While it is clear that the nature of the cohesive sites is important to an understanding of the vegetative-prophage transitions depicted in Figs. 1 and 2, what is more relevant to our present subject is the fact that by a simple heat treatment (19), solutions of lambda or lambda dg DNA can be obtained in which essentially all the molecules exist as the open monomer. Such solutions constitute the starting material used in the experiments described in the succeeding sections.

The Gene Content of the Two Halves of Lambda dg DNA A few years ago John Simmons and I (22) investigated the distribution of genes in a population of fragments of lambda dg DNA which we called "half-molecules" or, more loosely, "halves." These fragments resulted from the application of hydrodynamic shear created by stirring solutions of open monomers of lambda dg DNA, molecules referred to here as "wholes." The evidence that led us to term these fragments "halves" came from the change in viscosity and in sedimentation coefficient attendant upon breakage and the fact that we could divide this set of fragments into two subsets that exhibited a 1 : 1 mass ratio (22)

The size distribution of the halves is more graphically portrayed in Fig. 3 which derives from recent measurements by Inman of the lengths of molecules in a sample of the halves and of the wholes. The most frequent length in the sample of halves is one-half the most frequent length in the sample of wholes. The asymmetry observed in the length distribution of the sample of halves indicates that some whole molecules received more than one break. This distribution is consistent with 75% of the wholes suffering a single break near the center (0.5 ± 0.15) and 25% suffering more than one break.

We were able to isolate two subsets from this set of halves (22). We call these the "left and right halves" because they contain the genes located on the left and right halves of the vegetative map. The first isolation of these two subsets was effected by chromatography on columns of methylated serum albumin adsorbed to kieselguhr (MAK columns).[1] The results of such an isolation are given in Fig. 4. The DNA eluting from the column (As_{260}) forms two peaks, which represent the left and right halves respectively. This is indicated in the figure by the distribution of two genetic activities among the fractions. Thus the activity for the *t* gene of the galactose operon (*gal*$^+$) located on the

[1] The basis for the fractionation is a difference in frequencies of the bases in the two halves of lambda dg DNA; the left halves have a higher frequency of *G-C* base pairs than do the right halves (22). This is also the case for the halves of normal lambda DNA (20, 22). As a consequence, isolation of each subset can also be effected by sedimentation to equilibrium in CsCl density gradients (20, 22); and, after reaction with Hg(II), in Cs_2SO_4 density gradients (24).

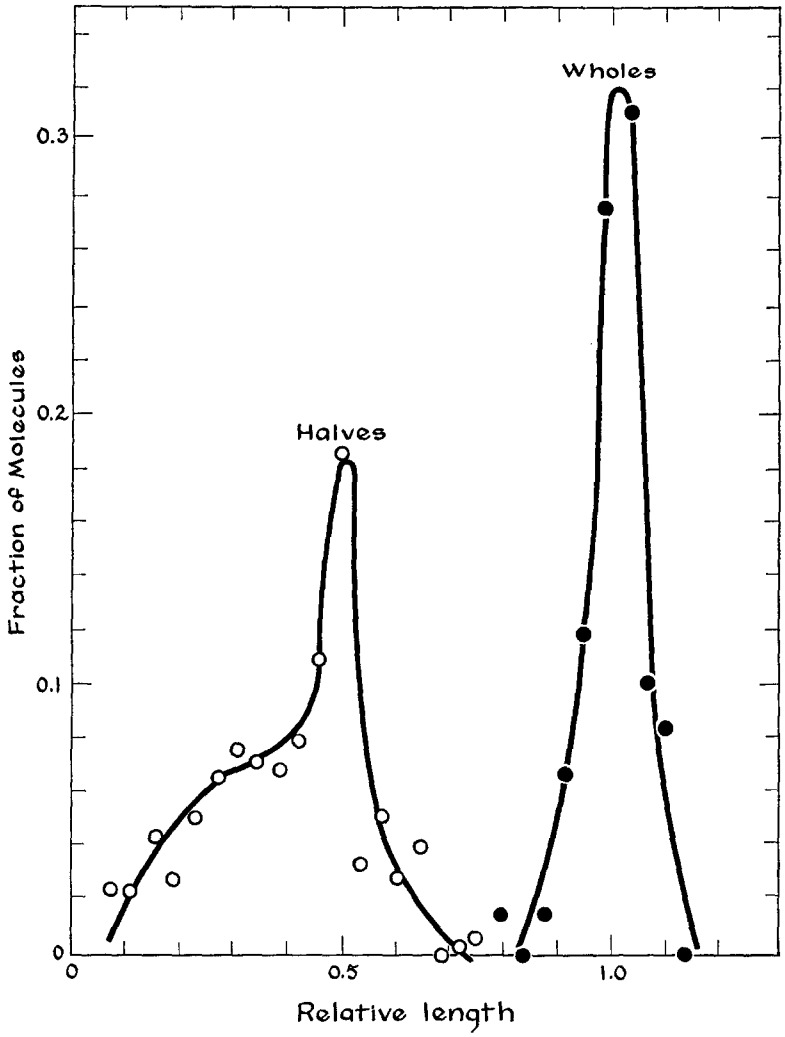

FIGURE 3. Length distribution of whole and half-molecules of lambda dg DNA. The relative lengths given on the abscissa are the contour lengths of the molecules observed in the electron microscope according to a modification (23) of the Kleinschmidt technique after normalization to a value of one for the number-average of wholes, which in metric units is 13.2 microns. Under these conditions, normal lambda DNA exhibits a distribution centering at 14.5 to 15 microns (4). The above population of lambda dg wholes represents 58 closed molecules. Six additional closed molecules were observed, but these had lengths between 24.5 and 28 microns (Number - average = 26.3 microns) and were assumed to be dimers. Closed, rather than open molecules were chosen to represent the distribution of wholes to avoid including fragments which contaminated this preparation of wholes (14% of total mass). The observed distribution of halves was corrected for these contaminating fragments in order that the above distribution represent those fragments resulting from breakage of wholes by the applied shear. The number- and weight-average lengths for the 128 molecules in the resulting distribution of halves are 5.2 and 5.9 microns, respectively.

left-hand half of the map is largely localized in the first peak eluting from the column, while the activity of a gene located on the right-hand half of the map (i^{λ}, see the legend of Fig. 4) is restricted to the second peak. When the peak fractions of each subset were examined for the activities of genes *A* (or *B*), *k*, *e*, and *R*, the genes *A*, *k*, and *e* were restricted to the DNA from the first peak, while *R* appeared only in the DNA of the second peak.[2]

Thus the results of these early experiments are consistent with the supposition that the gene distributions on the map and in the DNA are colinear. Fragmentation experiments with the DNA from normal lambda similar to the above but not involving isolation of the two types of half-molecules have also yielded results consistent with colinearity (31, 32). However, because the resolution is at the extremely crude level of half-molecules, this consistency is associated with a low order of significance. For example, the results are compatible with colinearity between the DNA and either the vegetative map or the prophage map of the phage (see Figs. 1 and 2). Quite clearly, breakage of the DNA into fragments smaller than halves and the analysis of their gene content are necessary.

The Position of the Genes in the Right Half of Lambda or Lambda dg DNA

Before considering our most recent experiments to locate more precisely the position of the genes in the DNA, I should first briefly explain how we measure the activity of genes within fragments of the DNA. The basic procedure was developed several years ago (33) and consists of allowing the DNA to react with cells of *E coli* K12 which have been made competent by a previous infection with helper phage. Thus, to assay for the activity of a given gene, call it α^+, on a fragment of DNA, we first infect the bacteria with a phage which is defective in regard to the function of this gene. These are the α^--helper phage and the defect in α-function may be due either to a mutation in the gene or to the absence of the gene. If the α^+-containing fragment of DNA enters the cell, recombination between it and the DNA of the helper phage can take place to yield whole molecules of lambda DNA containing α^+. Such DNA can either become prophage creating a lysogenic bacterium, or it can be replicated in the vegetative state to become mature phage that is released upon cell lysis. These mature α^+-phage can be scored as plaques by plating with appropriate bacteria.

In the case of the phage genes, it is the plaque-producing response that we assay. When, however, α^+ represents a gene of the galactose operon, then the assay largely depends upon the lysogenic response of whole lambda dg DNA created by recombination between a fragment derived from lambda dg DNA

[2] The mutant sites actually investigated were m_6, k_8, e_{22}, and *mi* (22). The m_6 site is in the *A* or *B* gene, or between the two (27). The *mi* site (28) is closely linked to the supressor-sensitive mutants used to define the *R* gene (6, 29) and, like these, specifically affects the synthesis of the lambda lysozyme to create abnormally low levels of its activity (30). The k_8 and e_{22} are specific sites in the *k* and *e* structural genes of the galactose operon (15).

and the DNA of non-dg helper phage. In this case the bacteria must also be α^- (i.e., galactose-negative) so that the result is the conversion of galactose-negative bacteria to galactose-positive bacteria which can easily be scored. Here it should be noted that in a small fraction of the cases it is possible that

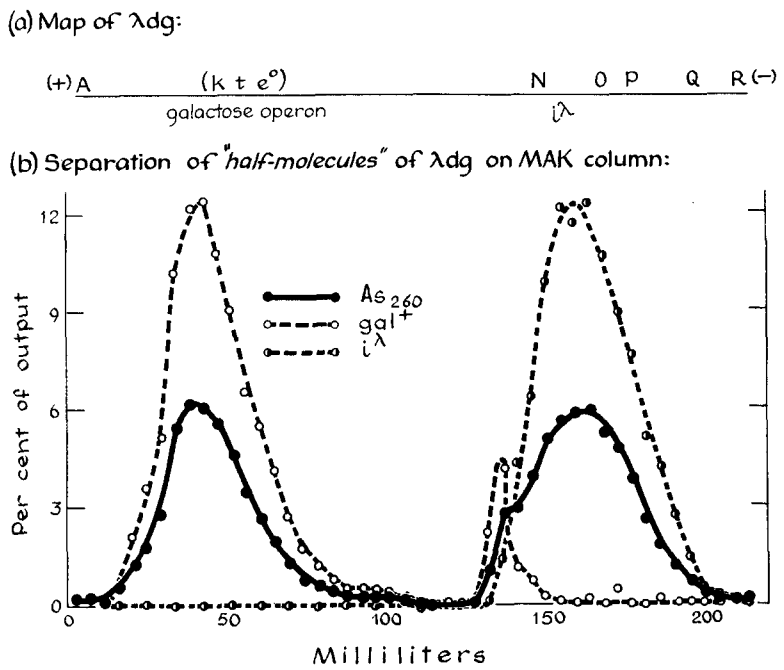

FIGURE 4. Isolation of left- and right-halves of lambda dg DNA. (*a*) Except for i^λ, the symbols are those given in Figs. 1 and 2 and defined in the text adjacent to these figures. The i^λ designates the region which specifies the immunity of the lysogenic bacteria (25). This region lies very close and to the right of *N*, with the possibility of some overlap (6, 26). (*b*) This figure is taken from Hogness and Simmons (22) which should be referred to for the detailed conditions of the experiment. The left-hand peak was eluted from the column of methylated serum albumin adsorbed to kieselguhr (MAK) at 0.52 M NaCl while the right-hand peak was eluted by applying a linear NaCl gradient from 0.520 M to 0.600 M starting at 110 ml. As_{260} represents absorption of the fractions at 260 mμ. The gal^+ and i^λ refer to the activity of the *t* gene of the galactose operon (see Fig. 1) and the activity of the i^λ region, respectively. *Figure reprinted by permission of the Journal of Molecular Biology from J. Mol. Biol., 1964, 9, 411.*

recombination of the gal^+ fragment with the chromosome of the gal^- bacteria might take place without previous recombination with the helper phage (22).

We wished first to locate more accurately the genes on the right half of the vegetative map—namely, *N*, *O*, *P*, *Q*, and *R* (Figs. 1 and 2). To do this we—and by "we" I mean Dr. Egan and myself—caused breaks to occur in the set of halves by increasing the applied hydrodynamic shear. In this manner a set

of fragments was obtained whose lengths were estimated from their rates of sedimentation to center about one-sixth the length of whole molecules. Similarly, by further increasing the applied shear to lambda DNA, a set of

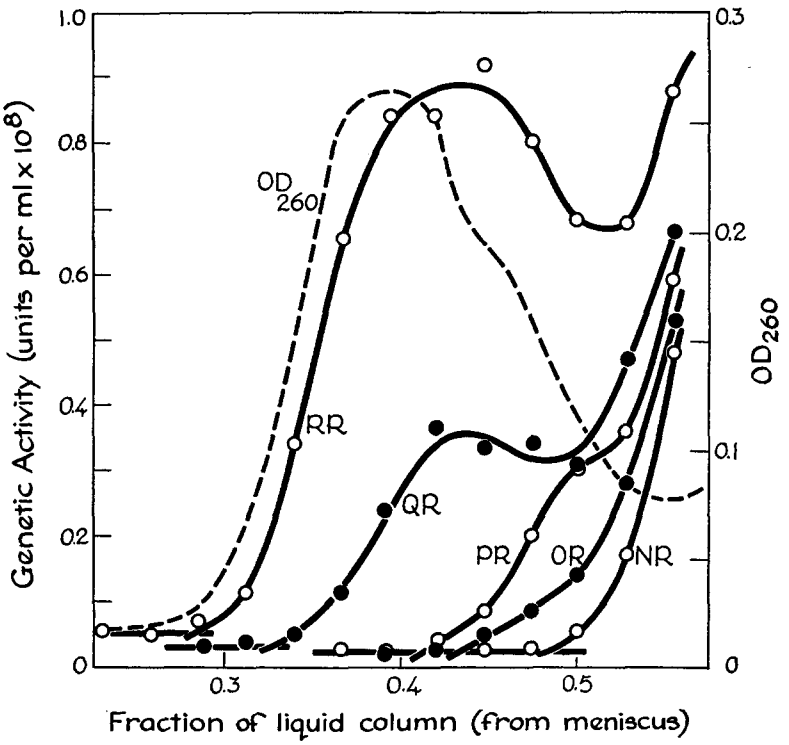

FIGURE 5. The distribution of DNA fragments according to their rates of sedimentation. The general method is described in the text. The centrifuge tube initially contained 4.6 ml of a solution of sucrose in 1 M NaCl, 0.01 M Tris-HCl buffer, pH 7.1, the sucrose concentration varying linearly with volume from 5 to 20% (w/v). 0.1 ml of a mixture of halves, sixths, and twelfths (see text) in a 1:1:1 mass ratio containing a total of 19 μg of DNA was layered on top and the tube then centrifuged for 7 hr at 38,000 RPM in a SW 39 rotor (Beckman-Spinco), 2°C. Fractions were collected and assayed for optical density at 260 mμ (OD_{260}) and for activity of the indicated gene pairs. The activity assay differs from that published (4) in respect to the helper phage and the recipient bacteria. The helper phage used in assaying the gene pairs were the double mutants N_7R_{60}, $O_{29}R_{60}$, $P_{80}R_{60}$, and $Q_{73}R_{60}$ where the subscript numbers refer to the *sus* mutants of Campbell (6). The *RR* curve in the figure designates the activity for the *R* gene independent of other genes, the helper phage being $R_{54}R_{60}$. The recipient and indicator bacteria were the nonpermissive W3350 except for the *N-R* assay in which a permissive strain, C600, was used as recipient (6).

The solution layered on the gradient also contained a small amount of purified *E. coli* β-galactosidase (gift of M. Cohn) which was used as an internal standard for calculating the sedimentation coefficient of the DNA (34). It sedimented as a single zone with a peak at 0.39 on the abscissa.

fragments with lengths centering about one-twelfth that of whole molecules was obtained. We then combined equal weights of the three sets of fragments loosely referred to here as "halves," "sixths," and "twelfths." The point here was to achieve a fragment population with a very wide distribution of lengths.

This population of fragments was subjected to zone sedimentation in a sucrose gradient, thereby spreading the fragments out along the axis of the centrifuge tube according to their sedimentation coefficient, and therefore, according to their size. The fractions collected from the tube were then assayed for the activity of the following gene pairs: *N-R*, *O-R*, *P-R*, and *Q-R*. The helper phage used in these assays contained a mutation in each gene of the pair so that a positive response indicates both genes of the pair are present in the active fragment. The fractions were also assayed for the presence of the *R* gene without demanding the presence of the other member by using helper phage mutant only for the *R* gene.

Since the order of these genes on the vegetative map is the dictionary order, *N*, *O*, *P*, *Q*, *R*, as one proceeds towards the right end, colinearity of map and DNA would demand that the smallest framgents containing both genes of a given pair would have the following size relationship for the various pairs: *N-R* > *O-R* > *P-R* > *Q-R* > *R* alone. The result of this experiment confirms this prediction and is given in Fig. 5.

The direction of sedimentation is toward the right and only the relevant portion of the liquid column is shown on the abscissa. The distribution of DNA mass (OD_{260}) in this region exhibits a broad peak consisting of the fragments from the sets of sixths and twelfths, the peak of halves being off to the right and not shown in the figure. Looking at the activity distributions of the gene pairs, one immediately perceives that the smallest active molecules containing a given doublet—*R-R*, *Q-R*, *P-R*, *O-R*, and *N-R*—increase in sedimentation coefficient, and therefore in size, as the map distance between members of the pairs increases.

Actually, three quantities are being measured in each case rather than the two of the indicated pair. This results from the tact that in these assays only those fragments which contain either of the two ends of the whole molecule are active (4). Presumably at least one cohesive site is necessary for activity. When this fact is combined with the above results, one must conclude that the required end is closest to *R* among the group of genes shown here. Thus the distance from the meniscus to the extrapolated position for each pair is indicative of the distance in the DNA between the required end and the non-*R* gene of the pair. We conclude that the order of the genes in the DNA is *N-O-P-Q-R*-end. This is the sequence found in the vegetative map, not in the prophage map (Fig. 1).

It should be noted here that the activity distribution of the i^{λ}-*R* pair (see Fig. 4) was also determined, though it was not included in Fig. 5 for the sake

of simplicity. The extrapolated position for this pair is located between that for *N-R* and *O-R*, though its position is not significantly different from that for *N-R*. This is also consistent with the genetic map.

We can make a rough estimate of the sedimentation coefficient of the smallest molecules active for a given pair. This is done by comparing the distance sedimented by such molecules (after allowing for the shape of a sedimenting zone of homogeneous DNA molecules) to the distance moved by a zone of homogeneous molecules of known sedimentation coefficient contained within the same centrifuge tube (34). The standard in this case was *E. coli* β-galactosidase which has a sedimentation coefficient of 16.2 Svedbergs.

From these admittedly rough estimates of sedimentation coefficient we can calculate the molecular weight of the respective DNA molecule (35), and from this value compute the number of included base pairs. The number of base pairs from the required end to the *R* gene is thus computed to be 3000, but it could be appreciably less than this number because we run out of DNA at the small end of the size spectrum. The *Q* gene appears between 4 and 5000 base pairs from the end; the *P* gene, between 8 and 9000; the *O* gene, a little farther along between 9 and 10,000 base pairs; and finally, the *N* (and i^{λ}) gene at about 13,000 base pairs from the end.

It should be emphasized that these numbers are first approximations. As such they do not differ significantly from the distances on the genetic map (6, 8–10). However, more accurate determinations of the molecular weight of the pertinent DNA molecules must be made before such a comparison is of much use. This we are doing.

The Order of the Galactose Genes in Lambda dg DNA In addition to these investigations on the position of genes in the right half, we have also determined the order of the genes of the galactose operon contained in the left half of lambda dg DNA. The order of these genes in the vegetative map of lambda dg has not been directly determined, but Campbell's model predicts that the order will be *k-t-e* as one proceeds from the left-hand end toward the center (Fig. 2). Using the principle that only fragments containing cohesive sites are active we have devised an experiment whose results confirm this order.

In this experiment, we take a solution of halves of lambda dg DNA and stir it at speeds sufficient to cause further breaks in the DNA so that eventually a population of fragments is obtained which have an average size about one-quarter that of whole molecules. During the transition from halves to quarters much of the activity for the genes of the galactose operon is lost. What we have measured is the rate of that loss for each of the genes *k*, *t*, and *e*. Consider the diagram and results presented in Fig. 6. The genes *k*, *t*, and *e* of the galactose operon are shown ordered on the left half of lambda dg DNA according to

Campbell's model (Fig. 2). What should happen when the set of such fragments is subjected to further breakage? All breaks to the right of the operator, *o*, should not destroy the activity of any of the galactose genes since such breaks do not divorce them from the required end. Similarly, all breaks to the left of *k* should inactivate all the galactose genes. These two classes of breaks should not differentiate among the three genes. However, breaks occurring

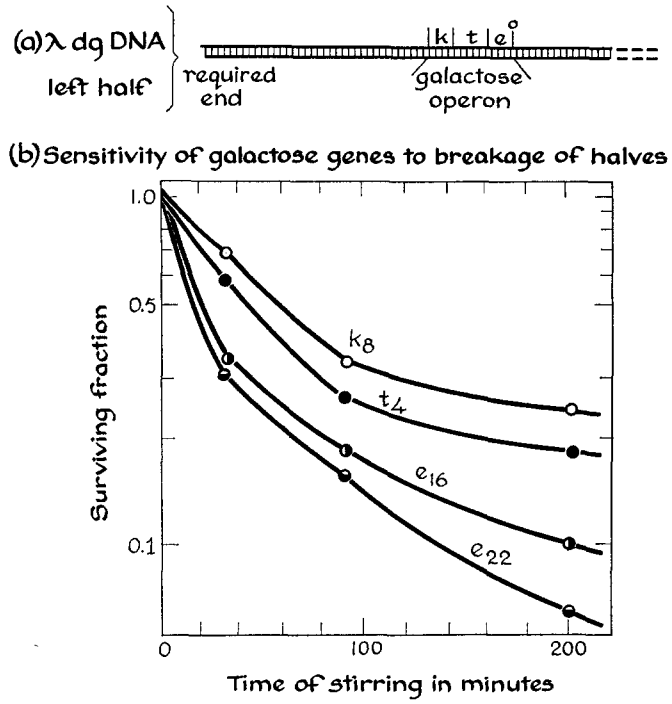

FIGURE 6. The sensitivity of galactose genes in halves of lambda dg DNA to further breakage. Halves of lambda dg DNA were stirred at 2,200 RPM in 0.001 M EDTA, 0.01 M Tris-HCl buffer, pH 6.7, according to the method of Hogness and Simmons (22) for the times indicated on the abscissa. The ordinate indicates the surviving fraction (log scale) of the activity for genes *k*, *t*, and *e*. The mutants k_8, t_4, e_{16}, amd e_{22} and the activity assays are described in reference 22.

within the operon should differentiate by destroying the activity of markers to the right but not to the left of the break point. The general prediction of this analysis is that among the linked gene activities in the set of halves, the farther the gene is from the required end the more sensitive will be its activity to further breakage. Thus if the order of the galactose genes given in Fig. 6 is correct, then the activity of *e* should be more sensitive to breakage than that for *t*, which in turn should be more sensitive than *k* activity.

The results of the experiment are given in Fig. 6 and clearly confirm the

prediction. The *k* activity is least sensitive and the *e* activity most sensitive, indicating that *k* is closest and *e* farthest from the required end. The subscript numbers refer to the particular mutants used in the assay and therefore to the specific region of each gene which must be contained in the active fragment. Two epimeraseless mutants, e_{16} and e_{22}, were used and the e_{22} appears to be more sensitive than the e_{16}. From this we would conclude that the e_{22} mutant site in the DNA is more distant from the *k* gene than is the e_{16} site. This is consistent with genetic recombination data of Adler and Kaiser (15) who found that e_{22} is farther from *k* than is e_{16} on the genetic map of the galactose operon as it appears in the *E. coli* chromosome.

It is of interest to note that the above analysis involves the supposition that the galactose genes remaining in active fragments resulting from breaks within the operon recombine with the galactose genes in the host chromosome. Were it otherwise, the galactose genes derived from the fragment would be separated from that sequence of base pairs at the operator end of the galactose operon which is necessary for its transcription. In this condition, the genes would be inactive.

From this brief summary of our data concerning the positioning of the genes on the DNA molecule isolated from the phage we come to the following conclusions. First, the genetic maps provide an accurate description of the order of the genes along the helical axis of the DNA molecules. Second, the ends of lambda DNA correspond to the ends of the vegetative map, not the prophage map. These two conclusions are also consistent with the data obtained by Kaiser and his associates (4, 31, 32) and by Jordan and Meselson (36). Finally, we have developed methods for specifying the position of a given gene on the DNA. At present, only a rough positioning has been effected. It appears, however, that the techniques are available for a rather precise positioning; for example, to within less than a thousand base pairs. It is now largely a question of how much work one wants to invest in the positioning of any given gene in lambda or lambda dg DNA.

THE ORIENTATION OF GENES IN LAMBDA dg DNA

The Basic Chemical Relationships Associated with Gene Orientation I should now like to turn from considering the position of the genes in the DNA to a consideration of their orientation. In illustrtating what I mean by gene orientation, I shall frequently refer to the N-to-C direction of a given structural gene. By this I mean the direction from the codon specifying the amino terminal residue of the polypeptide derived from the gene to the codon specifying the carboxy-terminal residue.

The N-to-C direction for the galactose genes in lambda dg DNA is indicated in Fig. 7 as "protein orientation." We do not know this by direct measurement; rather, it is based on the assumption that operons are similarly

oriented relative to their operator ends and on data obtained from the tryptophan operon in *E. coli*. These data indicate that one of the structural genes in the operon (that for the A protein of tryptophan synthetase) is so oriented that the amino terminal codon is closest to the controlling or operator end of the operon (37–40). There is appreciable evidence that the entire tryptophan operon is transcribed onto a single messenger RNA molecule (41, 42). This indicates that the other structural genes in this operon are oriented as is that for the A protein. Assuming that this orientation with respect to the operator also holds for the structural genes of the galactose operon, one obtains the N-to-C orientation shown in Fig. 7.

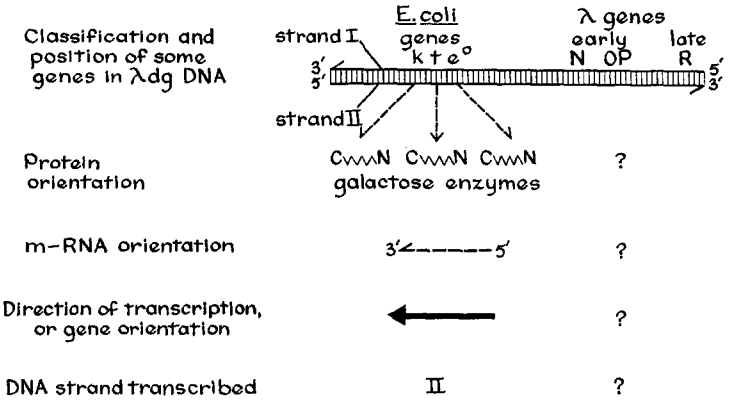

FIGURE 7. The orientation of genes in lambda dg DNA.

There is ample evidence from studies in vitro (43, 44) and in vivo (45) that translation proceeds in a 5′-to-3′ direction along the messenger RNA. Since the amino terminal residue is translated first (46), this means that the orientation of the messenger RNA for the galactose operon must be as shown, with its 5′-to-3′ direction corresponding to the N-to-C direction.

Recent data (47, 48) indicate that the synthesis of RNA catalyzed by *E. coli* RNA polymerase proceeds by chain extension at the 3′-end. Thus the direction of transcription should be the same as the 5′-to-3′ direction in the messenger RNA, which, as we have seen, is the same as the N-to-C direction. This direction defines the orientation of a structural gene. In Fig. 7, the galactose genes in lambda dg DNA are oriented from right to left.

Finally, consider which of the two antiparallel strands of the DNA (strands I and II in Fig. 7) provides the template for the galactose operon. When a single strand of DNA provides the only template for RNA synthesis catalyzed by RNA polymerase in vitro, the RNA product and the DNA template can form a duplex that appears to have a structure like that of double-stranded DNA, with the bases paired according to the Watson-Crick rules and the

5′-to-3′ direction of the RNA being antiparallel to that of the DNA (49–52). Thus we speak of the RNA product being antiparallel to the single DNA template strand.

When double-stranded DNA provides the template, the product RNA molecule appears to be complementary and antiparallel to part of one of the DNA strands (52–54). The now classical assumption is that this complementary antiparallel region in the DNA strand is the template for the synthesis of the given RNA molecule. I make that assumption here, but also note that it has yet to be demonstrated, a subject that I shall return to at the end of this paper. On the basis of this assumption, the strand of lambda dg DNA that is the template for the genes of the galactose operon is strand II (Fig. 7).

The above analysis indicates that there are three alternative ways of determining the relative orientation of two genes located on the same duplex DNA molecule: (*a*) determination of the relative N-to-C directions, (*b*) determination of the relative directions of transcription, and (*c*) determination of whether the same or different strands of the DNA function as template. Methods are available for the first and third determinations and I shall emphasize the third here.

Questions of Gene Orientation in Lambda DNA A comprehensive description of a DNA molecule at the level of individual genes should include not only the position of each gene in the DNA, but also its orientation. From such a description one might hope to discern the general rules, if any, which govern gene orientation.

The experiments of Marmur and his associates (55) along with those of Geiduschek's group (56) were initially interpreted to indicate that only one strand of the duplex DNA from certain bacteriophage (SP 8 and alpha) was transcribed in the *B. subtilis* host; i.e., that transcription was unidirectional. This stemmed from the ability to isolate from the denatured phage DNA two populations of molecules which were thought to represent the two strands; a mixture of the two populations could be renatured to yield duplex molecules while this did not occur within a single population. The fact that significant amounts of RNA-DNA hybrid molecules could be formed from the reaction of one, but not the other, of these DNA populations with the RNA made during phage infection of *B. subtilis* formed the basis for the preceding interpretation. However, the argument is incomplete. The molecules within each population were considerably smaller than expected for complete single strands (55); thus different molecules in a single population might derive from different strands. Furthermore, the techniques used tended to estimate the fraction of RNA molecules which could combine with one or the other DNA population. Since different genes within the phage DNA may be transcribed at different rates, such fractions do not necessarily represent the fraction of genes which use molecules in one or the other population as template.

Some recent information concerning the DNA of bacteriophage T4 indicates that in this case transcription proceeds in different directions for different genes. Thus Streisinger (57) has found that the N-to-C direction within the structural gene for the lysozyme of T4 is opposite to that found by Brenner and his associates (58) for the gene determining the T4 head protein. Thus at this preliminary state in the analysis of gene orientation it is clear that we do not have an adequate description of gene orientation within lengths of DNA larger than that encompassing an operon to even begin the formulation of general rules.

In initiating a description of this sort for lambda DNA, we have first considered representatives from each of three classes of genes associated with lambda. The first class, genes of *E. coli* closely linked to prophage lambda, has already been discussed, using the galactose genes as representatives. The other two classes derive from the lambda genome and are differentiated on a temporal basis. The expression of each gene in the first class, termed *early*, appears to be a prerequisite for the expression of the genes in the second class, termed *late*. As is indicated in Fig. 7, *N*, *O*, and *P* are members of the early class, while *R* and genes *A* through *M* are late genes. The mechanism of the clock that determines the *early* and *late* functions is not understood but it would seem to be related to the replication of lambda DNA since experiments of Radding (59), of Dove (60), and of Brooks (61) indicate that expression of early genes is necessary for vegetative replication of the phage DNA, while expression of *late* genes is not.

These classes were chosen because of our interest in constructing molecular models for the regulation of gene expression in both the vegetative and prophage states of lambda and the clear relevance of gene orientation to such constructions. The gene, or genes, that determine the specificity of immunity (i^{λ}-region, see Fig. 4 *a*) obviously should be included as well, but as we have not yet attempted to determine their orientation, they are not emphasized here.

We have selected one gene from each group, *early* and *late*. Black, in our laboratory, is in the process of ascertaining the N-to-C direction for gene *R*, which determines the structure of the lambda lysozyme. He has isolated this polypeptide chain containing some 165 amino acid residues and has divided it into three ordered subpeptides by reaction of cyanogen bromide with its two internal methionine residues. However, the corresponding subpeptides of lambda lysozymes from *R* mutants (62) have yet to be analyzed for altered amino acid residues. One of the subpeptides is quite small (13 residues), while the other two are about 4 and 8 times larger (63). Consequently, we do not anticipate difficulty in finding two *R* mutants whose affected residues lie on opposite sides of either internal methionine, and thus in determining the

N-to-C direction. However, at the present time, the N-to-C direction for this, or any other lambda gene, remains unknown.

It is clear that determination of the N-to-C direction for the various lambda genes is a formidable task, particularly when it is recognized that the proteins determined by most lambda genes have not been identified, let alone purified. We should like a method independent of a knowledge of the primary structure

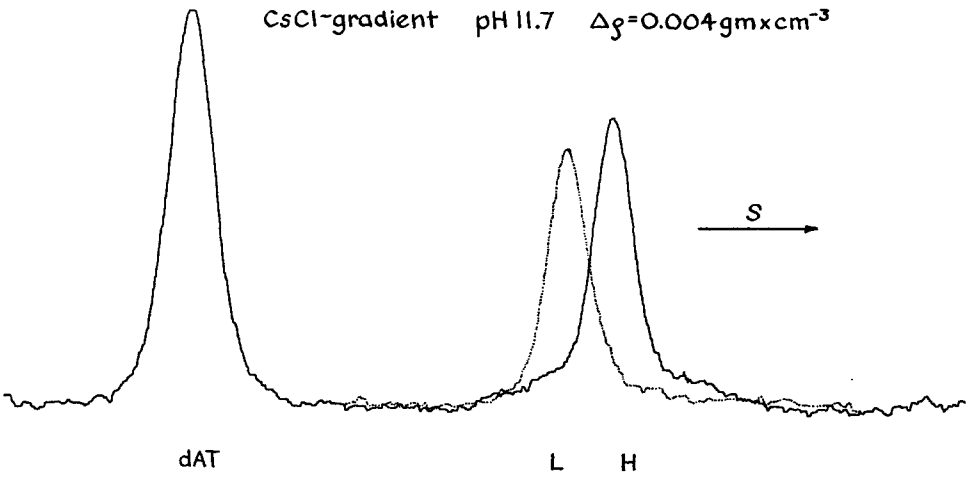

FIGURE 8. Sedimentation to equilibrium of the isolated strands of lambda DNA in CsCl density gradient. The figure represents the combined results from two different experiments. Both centrifuge cells contained polydeoxy AT (gift of A. Kornberg). One cell contained isolated *L* strands and the other isolated *H* strands. The cells were centrifuged in Spinco Model E ultracentrifuge for 20 hr at 23°C and at 44,770 RPM before ultraviolet absorption photographs were taken. After scanning each photograph with a Joyce-Loebl recording microdensitometer, the dAT peaks of the resulting tracings were aligned to obtain the above graph. The cells were filled with 0.7 ml of the following mixture: 0.55 ml of *H* or *L* strands in 0.001 M EDTA, 0.01 M Tris-HCl buffer, pH 7.1 (OD_{260} − 0.02); 5 μl of dAT copolymer in 0.15 M NaCl, 0.015 M sodium citrate, pH 7 (OD_{260} = 2.7); 10 μl of 0.1 M sodium EDTA, pH 7; 25 μl of 0.5 M sodium phosphate buffer, pH 11.7; 5 μl of 1 M NaOH; and solid CsCl to $\rho_{25} = 1.739$. The difference in density between the two strands ($\Delta\rho$) was calculated from the equation ($d\rho/dr = \omega^2 r/\beta$) given by Ifft, Voet, and Vinograd (65) in which β was taken to be 1.20×10^9.

of the relevant protein and, to this end, have emphasized the determination of which strand functions as template for a given gene. At this initial stage we have determined this strand for one of the early genes, *N*.

The Two Strands of Lambda DNA The question of which strand functions as template necessitates their separation and isolation. This we have done (64). I do not wish to describe here the isolation procedure which Doer-

fler developed in our laboratory; rather I should like to summarize some of the properties of the isolated strands.

The property which forms the basis of their isolation is the different buoyant density they exhibit when centrifuged to equilibrium in an alkaline CsCl gradient. This is shown in Fig. 8. Here the results from two separate centrifugations are superimposed. The reference band is formed by dAT copolymer which was present in both centrifugations, each of which also contained one but not the other of the isolated strands. We term the strand with the lowest density, *L*, and that with the highest density, *H*. The difference in density of 0.004 $g \cdot cm^{-3}$ is most probably due to a difference between the strands in the

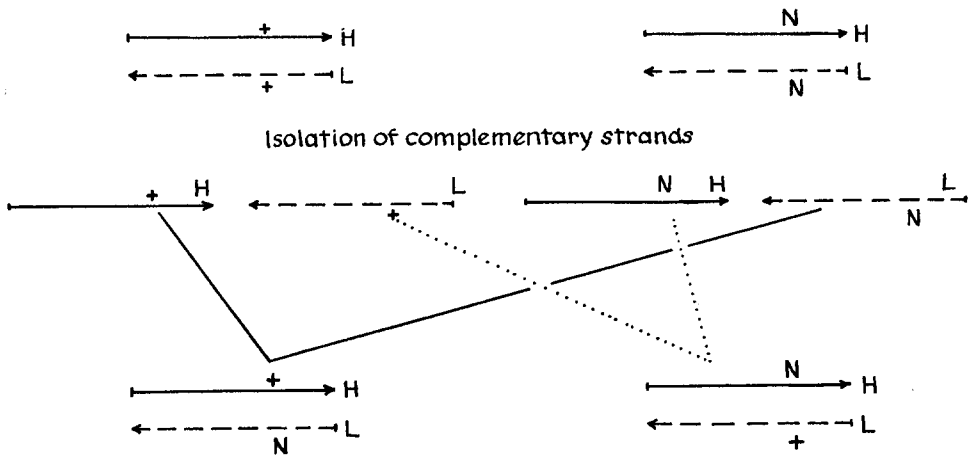

FIGURE 9. Scheme for the construction of heteroduplex molecules $+/N$ and $N/+$.

sum of their guanine and thymine residue frequencies. These are the two base residues in DNA which lose a proton as the pH is raised above ten, thereby causing additional Cs^+ ions to associate with the DNA and a consequent increase in buoyant density which is dependent upon these base frequencies. The difference in buoyant density of 0.14 $g \cdot cm^{-3}$ that we have found for the two strands of poly d-TG:AC is the extreme example of this effect (66).

The preparations of each strand are contaminated by less than 1% of the complementary strand. Their sedimentation patterns and coefficients indicate that they consist of the complete strand, being reasonably free of fragments. In our assay system for genetic activity, the single strands have no significant activity. However, when equal amounts of each strand are mixed and subjected to renaturation conditions of pH 10.5 and 37°C, genetic activity is regained for all genes that we have tested. The active molecules in such renatured material are identical to untreated native lambda DNA in regard to their sedimentation coefficient and buoyant density in neutral CsCl.

The Formation and Activity of Heteroduplex Molecules To answer the question of which strand acts as the template for gene *N*, we first construct some special molecules of lambda DNA which are called heteroduplex molecules. Their definition and construction are illustrated in Fig. 9. Consider the molecules illustrated in the first row. One is the DNA from normal wild-type lambda while the other contains a mutation in gene *N*. Thus the mutant molecule differs from the wild-type by the change of a base pair at the indicated position. To form the heteroduplex molecules, the single strands from each of these two DNA's are isolated, giving us the four single strand prepara-

TABLE I
ACTIVITY OF GENE *N* IN HOMO- AND HETERODUPLEX MOLECULES

Type of DNA	Relative activity of gene *N*
Homoduplexes	
H^+/L^+	1.00
H^N/L^N	<0.01
Heteroduplexes	
H^+/L^N	0.6
H^N/L^+	0.5

The *N* mutant used to form the above DNA molecules is sus_7 obtained from Campbell (6). The homo- and heteroduplexes were formed as indicated in the text and then assayed for *N* and *R* activity according to the legend of Fig. 5. Recipient and indicator bacteria were the nonpermissive W3350 (6). The helper phages were sus_7 and the double *R* mutant $sus_{54}sus_{60}$ when assaying for *N* and *R* activity, respectively. The values given in the table represent the average ratio of *N* to *R* activity for each DNA relative to that for the H^+/L^+ DNA, the values from single assay sets lying within ±25% of these values.

tions shown on the second line. There are four possible ways to pair these four single strands if each pair must contain an *H* and an *L* strand. Two of these yield the original homoduplex structures. The other two are shown in the last line and are called heteroduplex structures. In these there is a mismatch of bases at the position of mutation.

Doerfler has separately made each of the heteroduplex molecules shown here simply by exposing the appropriate mixture of two strands to the renaturation conditions mentioned previously. In terms of their physical properties of sedimentation coefficient and buoyant density, they are the same as the original homoduplex molecules. The question of interest here, of course, concerns the activity they exhibit for gene *N*.

I have mentioned the fact that expression of gene *N* is necessary for normal replication of lambda DNA. We rely on this property in the design of the heteroduplex experiment. Thus it is presumed that gene *N* must be transcribed to yield messenger RNA and then active enzyme before replication of

the DNA can take place. The N mutant we are using is a conditional lethal of the amber type. The mutant is not active in certain strains, called nonpermissive, whereas wild-type lambda is active in these strains (6). Since we use the nonpermissive strain in our assay, infection by the homoduplex DNA from the N mutant should not lead to replication of the DNA and release of phage—which it does not. Now consider the case when the nonpermissive cells are infected with the heteroduplex DNA's. If the H strand is that transcribed for gene N, then one should expect the H^+/L^N heteroduplex to yield wild-type messenger RNA from the wild-type sequence in the H strand and therefore to be active. By the same token the H^N/L^+ heteroduplex should yield mutant RNA and be inactive. Reciprocal results would be predicted if it were the L strand which is transcribed.

The activities that were found for both heteroduplexes are given in Table I, along with those for the two homoduplex molecules. The homoduplex molecules give the expected results, but the values obtained for the heteroduplex molecules are clearly different from those predicted.

Possible explanations for the fact that both heteroduplex molecules are active can be divided into two classes. In one, it must be supposed that the wild-type messenger RNA can somehow be synthesized using either heteroduplex as template. We initially rejected this class of working hypotheses as not only heretical but also implausible and, as you shall see, have not had reason to return to them. In the second class of explanations, one assumes that the wild-type homoduplex DNA is formed from either heteroduplex prior to synthesis of the wild-type messenger RNA.

In considering this class of explanations we noted that the activity of either heteroduplex is less, in fact approximately one-half that of the wild-type homoduplex molecules. In respect to these relative values I should mention that variations in efficiency of renaturation in the formation of the different molecular types have been taken into account. The renatured wild-type homoduplex preparations usually contain from one-third to two-thirds the genetic activity of untreated wild-type DNA. This variation is accounted for when comparing the different preparations by measuring the activity of another gene, in this case gene R, for which the four molecular forms given in Table I contain the same wild-type structure. The values given in Table I have been normalized with respect to these R activities.

We assumed that the lower values for the heteroduplexes were significant and constructed a model to account for it. More important, this construction led to an experiment which I believe does determine the strand that functions as template for gene N. The model is illustrated by the diagram shown in Fig. 10. The two heteroduplex molecules are represented on the first line. The position of mismatch of the bases is indicated by the separation. It is supposed that this substitution causes a local alteration of structure such that the affected

region becomes subject to the excision and repair mechanisms which operate on DNA-containing lesions caused by ultraviolet irradiation (67–69). The first step in the process is imagined to be excision, and here the simplest supposition is that the probability of excising from one strand equals that for the other. The repair synthesis will then yield a homoduplex molecule that is either wild-type or mutant, this being the result regardless of which heteroduplex is considered. Since an active molecule is created in only one-half of the cases, the average activity should approach one-half that exhibited when starting with homoduplex molecules.

In the case of the heteroduplex containing the wild-type sequence in the template strand, the value would be expected to be greater than one-half if

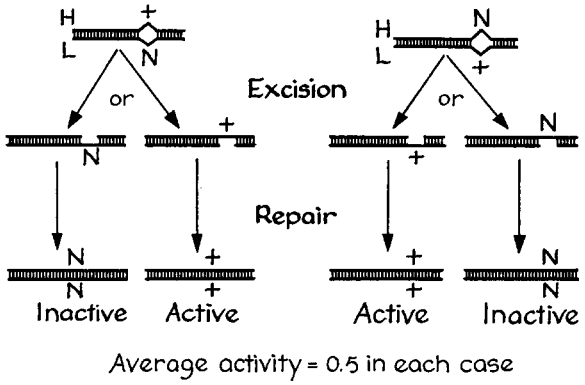

FIGURE 10. Model for the formation of homoduplexes from heteroduplexes by excision and repair.

transcription of gene *N* and consequent normal DNA replication to produce both homoduplexes were to win out against the competing repair mechanism.

Using this model as a working hypothesis we designed the following experiment. We argued that if we irradiated the cells used in the assay with increasing doses of ultraviolet light, we should be able to create a sufficient number of lesions in the DNA of the host cell to trap the enzymes involved in repair mechanisms. If the heteroduplex molecules should enter such a cell, the probability of forming the homoduplexes from them by the excision and repair mechanism would be greatly reduced. Consequently one would predict that the activity of the heteroduplex containing the mutant sequence in the template strand would be greatly reduced over the heteroduplex with wild-type configuration in that strand.

The predictions from this line of reasoning did in fact obtain when the experiment was performed, as is shown in Fig. 11. In this experiment, the cells were exposed to different doses of ultraviolet light before being infected with helper phage. The survival of competence for the wild-type homoduplex DNA

is shown in the upper part of the figure. Competence is a property of the cells that is relatively resistant to ultraviolet light. For example, after 250 sec of of irradiation the survival of colony formation is only 2×10^{-5}, whereas competence is little changed.

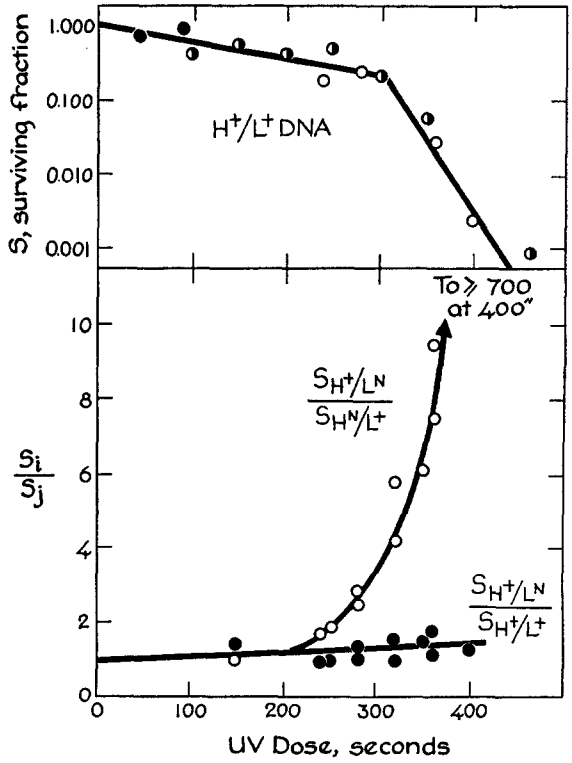

FIGURE 11. The effect of ultraviolet irradiation of the recipient cells on *N* activity The nonpermissive cells (W3350) were irradiated for the times indicated on the abscissa just before the addition of helper phage. (See table I for the assay of *N* activity.) The source of ultraviolet light was the General Electric germicidal lamp and the intensity at the surface of the culture was 17 ergs·mm^{-2}·sec^{-1}. The upper part of the figure represents the results of three different experiments in which H^+/L^+ DNA was assayed for *N* activity. The ordinate represents the surviving fraction of this activity as the recipient cells receive greater doses of ultraviolet light. In the lower part of the figure the ordinate is the ratio of the surviving fraction obtained with one DNA (S_i) to that obtained with another DNA (S_j), both DNA's being assayed with the same irradiated culture.

Rather than give a survival curve for each DNA, the ratio of the surviving fractions for pairs of DNA's is given in the lower part of the figure. Of primary interest is the survival ratio of the two heteroduplex DNA's, H^+/L^N to H^N/L^+. This ratio does not vary markedly until the dose is increased above 250 sec; then there is a dramatic rise such that the ratio reached a value greater than

or equal to 700 at 400 sec. Thus at this dosage there is an essentially qualitative difference between the heteroduplexes; the H^+/L^N is active and the H^N/L^+ is not. We interpret this to mean that the H strand is the template strand for gene N.

That the striking differential effect of ultraviolet irradiation of the cells on genetic activity of the heteroduplexes is specific to gene N is indicated by a control experiment shown in Fig. 12. The experiment is the same as the previous one, except that the activity of gene R is measured. The helper phage is an R mutant which can supply the N function if need be. Thus the only gene that is measured is R and each of the types of DNA contains the wild-type homoduplex structure for this gene. There is no significant differential effect of irradiation on any of the three DNA's tested, the homoduplex and the two heteroduplexes. Thus the differential effect of ultraviolet irradiation of the cells is not on the whole heteroduplex molecules, but rather only on gene N in those molecules.

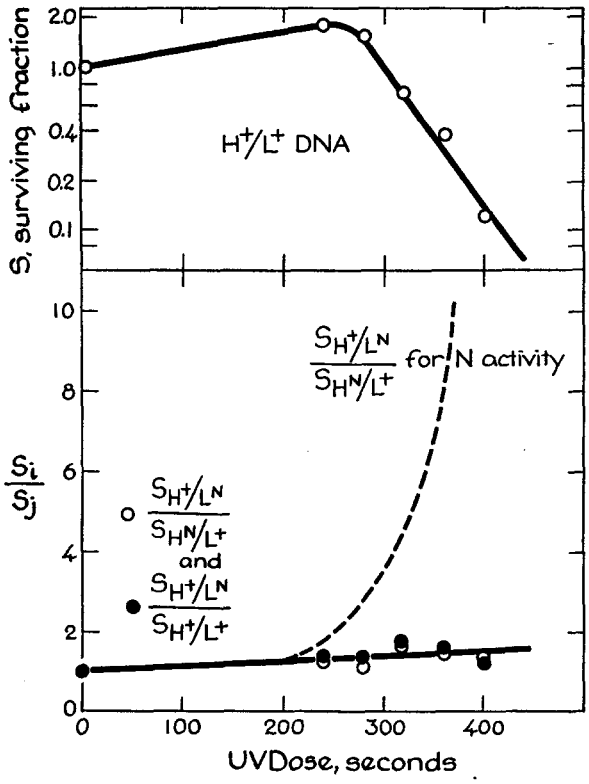

FIGURE 12. The effect of ultraviolet irradiation of the recipient cells on R activity. The methodology is the same as that indicated for Fig. 11 except that the R activity was measured (see Table I for the assay).

CONCLUSION

I want to emphasize that we have only begun the description of gene orientation in lambda by pointing out two parts of the problem which we have not yet solved and are working on now. The first is obvious from the manner in which I have presented this work. The technique of ascertaining which is the template strand will determine the relative orientation of two genes if we know that strand for each gene. However, if the orientation for one of two genes is determined by the N-to-C direction, whereas for the other gene the template strand is known, then one cannot relate the directions of transcription until the 3′-to-5′ direction of the strand is known. This is indicated in Fig. 13.

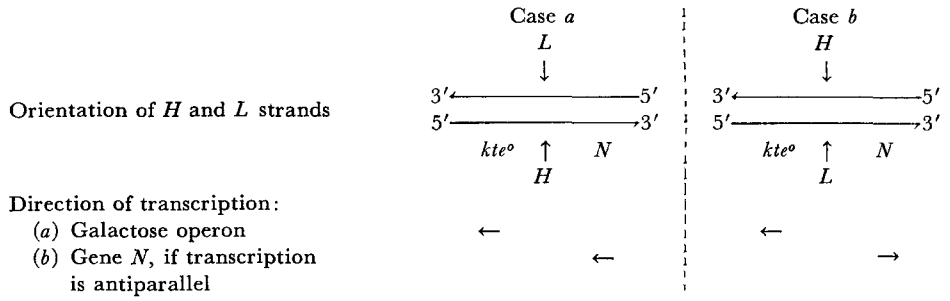

FIGURE 13. The relationship between the orientation of the *H* and *L* strands and the direction of transcription for gene *N*.

Consider the two cases shown; the *H* strand is assumed to be oriented either with its 3′-end nearest gene *R* (case *a*, where *H* equals strand II of Fig. 7), or with that end nearest gene *A* (case *b*). Since it is the *H* strand that is transcribed for gene *N*, then if transcription proceeds in an antiparallel sense the direction of transcription for this gene will be opposite to the 5′-to-3′ direction of the *H* strand. In case *a*, the direction of transcription would be the same for gene *N* as it is for the galactose operon, while in case *b*, the directions would be opposite.

We are attempting to determine the orientation of the strands by treating an isolated strand (*H* or *L*) with *E. coli* exonuclease I which attacks only the 3′-end of the strand (70). After such a treated strand is recombined with an untreated complementary strand, a duplex molecule will be formed in which only one end is altered. There are several ways of testing whether this is the right- or left-hand end and we are presently in the midst of these tests.

Finally, the determination of orientation depends upon the previously mentioned assumption that transcription proceeds by antiparallel movement along the strand that is transcribed. We have, within the experiments I have described, the possibility of determining whether antiparallelism is the case,

or whether transcription of the duplex DNA involves the alternative of the messenger RNA running parallel to the template strand. Thus for gene *N* we know that *H* is the template strand. If we could isolate the messenger RNA for gene *N* and determine which strand would react with it to form a DNA-RNA hybrid, we could solve the problem. If transcription is antiparallel, this RNA should react with the *H* strand but not with the *L* strand. If, on the other hand, the direction of transcription runs parallel to the template strand, then the RNA should react with the *L* strand. Whether we can complete this experiment obviously depends upon whether we can isolate such specific messenger RNA molecules. The reagents we plan to use in isolating these RNA molecules consist of a catalogue of fragments of lambda DNA, all of which have the same end but vary in length, a catalogue which Dr. Egan is presently collecting. Thus the success of this experiment rests upon the resolution we can obtain within the catalogue and how frequently the gene orientation shifts, if at all, as one proceeds down the DNA.

This then is the present status of what we know and what we plan concerning gene position and orientation in lambda and lambda dg DNA. It is clear that, thus far, we have only scratched the surface

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Discussion

Dr. Hurwitz: I would just like to make some comments with regard to the direction of reading of lambda DNA, both in vivo and in vitro. Dr. Skalka, working in collaboration with Dr. Hershey, has obtained evidence that the AT-rich half of lambda DNA appears to be copied quite early during in vivo transcription. In addition, Dr. Cohen of our laboratory also has obtained evidence from in vitro transcriptions studies with RNA polymerase that the AT-rich half is preferentially copied during RNA synthesis.