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Gene Orientation in Bacteriophage Lambda as determined from the Genetic Activities of Heteroduplex DNA formed *in vitro*

WALTER DOERFLER† AND DAVID S. HOGNESS

Department of Biochemistry, Stanford University School of Medicine
Palo Alto, California, 94304, U.S.A.

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The orientation of the *N* gene in λ DNA has been determined to be from right to left by identifying the strand which functions as template during its transcription. This identification depended upon the separate construction *in vitro* of the two reciprocal heteroduplex molecules in which one strand contains the wild-type base sequence and the other contains the sequence of an amber mutant of *N*. Since amber mutants of *N* prevent replication of λ DNA in unsuppressed hosts, it was anticipated that only one heteroduplex would be active in unsuppressed cells, that one with the wild-type sequence in the template strand. However, both heteroduplexes are active. On the supposition that a conversion of heteroduplexes to homoduplexes occurs in these cells by a mechanism of excision and repair akin to repair of ultraviolet photoproducts in DNA, ultraviolet-irradiated cells were tested for activity. Conditions were found in which only one heteroduplex is active.

The results of experiments with heteroduplexes which include double mutants of *N* are consistent with the model of conversion by excision and repair, and confirm the orientation derived from the behavior of single heteroduplexes. The heteroduplexes containing a single amber mutant in the *O* gene (also required for DNA replication) were investigated, but the orientation could not be determined since the heteroduplexes are equally active under all conditions tested. An extension of the model to excision and repair associated with recombination offers certain advantages in co-ordinating the results of all three pairs of heteroduplexes.

1. Introduction

Transcription proceeds by the addition of nucleotide subunits to the free 3′-hydroxyl at one end of the growing RNA chain (Bremer, Konrad, Gaines & Stent, 1965; Maitra & Hurwitz, 1965). This RNA is presumed to be anti-parallel to that strand in the duplex DNA which acts as template; hence the direction of transcription is opposite to the 5′-to-3′ direction of the template strand. We define the orientation of a transcripton‡ as the direction of its sensible transcription. Thus each transcripton has but one orientation. However, because the strands of duplex DNA are anti-parallel, two transcriptons in the same molecule may be oppositely oriented.

† Present address: The Rockefeller University, New York, N.Y. 10021, U.S.A.

‡ Transcriptons are defined as the units of transcription and may be mono- or multigenic. Operons are one class of transcriptons, as the term transcripton does not imply a particular mechanism for the regulation of its transcription, whereas operon does.

The orientation of a given transcripton can be determined if its template strand can be identified and if the 5′-to-3′ direction of that strand is known. In the preceding paper (Doerfler & Hogness, 1968), we described a method for isolating each of the two strands of the DNA from bacteriophage λ. The method depends upon the difference in buoyant density exhibited by the two strands in alkaline cesium chloride, presumably due to a difference in their GT contents†. The strand with the higher density is termed H, the other L. Using this technique for separating the strands, Hogness, Doerfler, Egan & Black (1966) and Wu & Kaiser (1967) identified the 5′-to-3′ direction of each strand by independent techniques. These directions and the position of some λ genes are given in Figure 1.

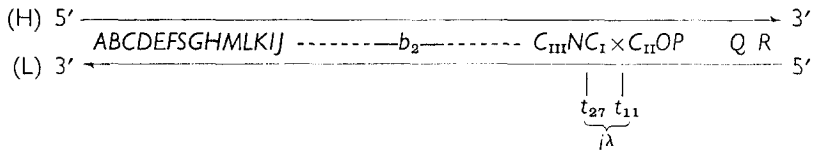


FIG. 1. The DNA of bacteriophage λ.

The H strand, which has the higher buoyant density in alkaline CsCl, has been termed W by Taylor *et al.* (1967) and the L strand termed C. The letters lying between the strands signify the various genes of λ. The positions in the DNA of *N*, i^{λ}, *O*, *P*, *Q* and *R* have been determined (Hogness *et al.*, 1966). C_I, C_{II}, C_{III}, *x*, t_{11} and t_{27} have been placed relative to *N* by their genetic map positions (Eisen *et al.*, 1966). The b_2 deletion constitutes about 17% of the DNA (Kellenberger, Zichichi & Weigle, 1961) and is located between *J* and C_{III} (Jordan, 1964). The order of the other genes is taken from Kayajanian & Campbell (1966), with *S* being added (L. Siminovitch, personal communication). Their position is approximated by allowing 1000 base pairs (2% of λ DNA) per gene and starting with *A* at 4% from the left end (Jordan & Meselson, 1965). Some of the characteristics of the mutants of these genes are given in the text.

In this paper we develop a method for identifying which strand functions as template for individual genes. The method is restricted to those genes the function of which is critical for DNA replication and is dependent upon the construction of specific heteroduplex molecules of DNA *in vitro*. The heteroduplexes are constructed from isolated intact strands of λ DNA, one strand derived from wild type and the other from a mutant of the gene in question. For a given mutation, two types of heteroduplex molecules can be constructed: H^+L^m and H^mL^+, where H and L designate the strands, and + and *m* indicate their wild-type and mutant origins (in specific cases, *m* is replaced by the symbol for the mutant gene). If the mutation blocks DNA synthesis, then only one of these heteroduplexes should be capable of yielding wild-type phage, i.e. be active. The active heteroduplex is the one with wild-type sequence in the template strand of the gene in question, and should thus be capable of generating the wild-type gene product without prior DNA replication. By the same argument, the wild-type gene product cannot be generated from the other heteroduplex prior to its replication, or more accurately, prior to the formation of the wild-type homoduplex from it. Thus by identification of the active heteroduplex, one should be able to identify the template strand and hence the orientation of individual genes belonging to the class necessary for DNA synthesis.

The early genes *N*, *O* and *P* belong to this class. Amber mutants of each have been isolated (Campbell, 1961) and shown to be defective in synthesis of λ DNA in unsuppressed hosts (Joyner, Isaacs, Echols & Sly, 1966; Eisen *et al.*, 1966). The *N* gene

† The abbreviations used here are defined in the previous paper (Doerfler & Hogness, 1968).

was chosen for first examination because it occupies a higher rank than *O* or *P* in the hierarchy controlling the transcription–translation of the λ genome, i.e. the expression of a greater part of the genome depends upon *N* rather than upon *O* and *P* (see Skalka, Butler & Echols, 1967; Taylor, Hradecna & Szybalski, 1967, for transcription data and for references concerning translation). In addition to the obvious reasons for interest in the more primary aspects of λ control, this basis for choice was employed to guarantee the necessary absence of λ DNA synthesis prior to expression of the given gene. The assays for synthesis of λ DNA used for definition of the mutant phenotypes of *N*, *O* and *P* were not sensitive enough to detect one round of DNA replication. We hoped that the greater deficiencies associated with the *N* mutants would render less likely that critical first round.

We shall describe experiments which fit the general plan given above and the results of which define the orientation of *N* as right to left in Figure 1. These results also indicate that the host cell contains systems for the conversion of heteroduplexes to homoduplexes, systems which can be saturated by exposure of the host cells to ultraviolet light and which are therefore assumed to be akin to systems for the repair of ultraviolet-induced lesions in DNA. We also describe similar experiments with the *O* gene. However, since no significant distinction between the heteroduplexes for this gene could be made, we suppose that the mutants of *O*, but not of *N*, provide their own system for heteroduplex to homoduplex conversion. A summary of some of these findings has been presented (Hogness, 1966; Hogness *et al.*, 1966).

2. Experimental Procedures

(a) *Materials*

Most media, chemicals, bacteria and phages used here have been described in the preceding paper (Doerfler & Hogness, 1968). The following items were not.

Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthridine-3-carboxylic acid) and 5-fluorodeoxyuridine were gifts from N. A. Goss (Sterline Winthrop Research Institute) and from Hoffman–La Roche, Inc., respectively.

The O_{29} mutant of λ was obtained by ultraviolet-induction of the lysogen, C600 ($\lambda i^{\lambda} O_{29}$) obtained from A. Campbell (1961). The double *R* mutant used for assay of this gene was obtained from C600 ($\lambda i^{\lambda} R_{54,60}$) isolated by A. D. Kaiser.

(b) *Methods*

(i) *General*

All of the methods used here except those given below have been described in the previous article (Doerfler & Hogness, 1968).

(ii) *Heteroduplex DNA*

Heteroduplex molecules of λ DNA were formed by mixing equimolar amounts of H and L strands, one isolated from wild-type DNA and the other from a mutant, under the renaturation conditions given previously for the formation of homoduplexes from isolated strands (Doerfler & Hogness, 1968). All single-strand isolates were assayed for purity by equilibrium sedimentation in alkaline CsCl gradients and by assay of genetic activity after exposure to renaturation conditions (see Doerfler & Hogness, 1968).

(iii) *Assay of λ gene activities with ultraviolet-irradiated* E. coli

The amber suppressor-negative strain, W3350, growing exponentially at 37°C in H-1, glucose (1·8 mg/ml.) medium, was harvested at $1{\cdot}1 \times 10^9$ cells/ml. by chilling to 0°C and centrifuging. The cells were washed once by suspension in I medium and sedimentation to a pellet, which was again suspended in I medium so that the optical density at 600 mμ ($O.D._{600}$) was between 3·0 and 3·2 (about $2{\cdot}1 \times 10^9$ cells/ml.). 8 to 10 ml. of this suspension in

9-cm Petri plates were irradiated with ultraviolet light from two General Electric germicidal lamps which gave an intensity of 15 to 17 ergs mm^{-2} sec^{-1} (measured with a Latarjet dosimeter) at the surface of the suspension located 61·5 cm below the lamps. During irradiation the plates were subject to continuous acentric rotation to mix the suspension, and samples of 1·5 to 4 ml. were transferred to light-tight flasks at various times. All subsequent operations on those samples were carried out in rooms having their lights off. The samples were shaken at 37°C for 10 min and then kept at 0°C for 5 min before being infected with the appropriate mutant helper phage (multiplicity of infection = 10) according to Kaiser & Inman (1965), whose procedure was followed for all subsequent steps in the assay. W3350 was used as the indicator bacteria.

3. Results

(a) *The* N_7 *heteroduplexes*

(i) *Formation and properties*

The individual H and L strands of λ (wild-type) and λ N_7 were isolated exactly as described in the previous paper (Doerfler & Hogness, 1968) to yield four separate strand preparations termed H^+, L^+, H^N and L^N. The four possible duplexes were constructed by mixing equimolar quantities of each member of the four pairs and subjecting the four mixtures to the renaturation conditions previously described for the formation of H^+L^+ homoduplexes (Doerfler & Hogness, 1968).

The specific activity of the renatured H^+L^+ homoduplex for the *N* gene was considered in some detail previously and under the conditions used here is about 50% that of native λ DNA (Doerfler & Hogness, 1968). When the *N* activities of the three other molecular types were compared to that for H^+L^+, the results shown in Table 1 were obtained. Some variation in specific activity of different renatured H^+L^+ preparations has been found (Doerfler & Hogness, 1968). We have sought to remove the effect of such variation when comparing these *N* activities by utilizing the specific activity of another gene as an indication of the efficiency of renaturation, remembering that all other genes should have the homoduplex configuration in each

TABLE 1

Activities of the N_7 *heteroduplexes*

DNA†	Specific activity of *N*‡ (normalized to H^+L^+) (%)	*N* activity/*R* activity‡ (normalized to H^+L^+) (%)
H^+L^+	100·	100
H^NL^N	<0·5	<0·5
H^+L^N	38	55
H^NL^+	41	49

† The various types of DNA were constructed by exposing equimolar mixtures of the appropriate isolated strands to conditions of renaturation that have been described (see Experimental Procedures section (b)).

‡ The specific activity is the number of plaque-forming units/ml. of DNA solution with O.D.$_{260}$ equal to unity. The assays for *N* and *R* activities have been described (see Experimental Procedures section (b)); the competent bacteria being the unsuppressed W3350 infected with either λN_7 or $\lambda R_{54,60}$ helper phage; plating bacteria were W3350. Duplicate plates were registered in each assay and at least two independent assays performed per DNA per gene activity. Average values are given, the range being within ±25% of the average.

of the four types of DNA. We measured *R* activity and used the ratio of *N* to *R* activity (Table 1) to take account of differences in renaturation.

The H^NL^N homoduplex behaves in the expected manner, exhibiting *R* activity but no significant *N* activity. The equivalent but reduced activities of the heteroduplexes are not the expected result if *N*-function is necessary for the conversion of heteroduplexes to H^+L^+ homoduplexes. This result cannot be accounted for by contamination of the H^+ or L^+ single-strand preparations with the complementary strand, since renaturation of each separately yields no more than 0·2% of the *N* activity of renatured H^+L^+, and no significant DNA banding at the density of duplex DNA in neutral CsCl gradients (Doerfler & Hogness, 1968). Nor can it be accounted for by assuming a small amount of *N* activity in the competent bacteria due to leakiness of the λN_7 helper phage, since essentially equivalent results were obtained when the double mutant, $\lambda N_{7,53}$, was used as helper. The efficiency of plating λN_7 on the unsuppressed recipient, W3350, is about 10^{-6}, while that of the $\lambda N_{7,53}$ is less than 10^{-9}. In the next section we consider experiments which indicate that it is the host cell itself which provides the activity for the heteroduplex-to-homoduplex conversion and present a method for elimination of this activity so that the two heteroduplexes exhibit grossly different *N* activities. Prior to such a consideration, we show here that the heteroduplexes exhibit sedimentation properties indistinguishable from renatured homoduplexes.

The equilibrium distributions of the H^+L^N and H^NL^+ heteroduplex preparations in neutral CsCl are given in Figure 2 along with that for the H^+L^+ homoduplex, shown again for comparative purposes. The buoyant density of the major peak in each distribution is 1·709 g ml.$^{-1}$; the minor peak represents some single-stranded DNA resulting from mixing amounts of H and L strands which are not exactly equimolar. As was indicated for the H^+L^+ distribution in the previous paper (Doerfler & Hogness, 1968), the shape of the major peak in each heteroduplex preparation is also somewhat asymmetric, being skewed toward the higher densities. When compared to native λ DNA, there is an excess of $15 \pm 5\%$ on the more dense side, this being in the range found for the renatured homoduplex.

When native λ DNA and the renatured H^+L^+ homoduplex were subject to zone sedimentation, the active molecules (*N* gene) in each preparation exhibited the same sedimentation coefficient of 34 to 35 s (Doerfler & Hogness, 1968). When the two heteroduplexes were separately sedimented in the same manner, the results shown in Figure 3 were obtained. As before, two standards, phage M13 DNA ($S_{20,w} = 30$ s) and *E. coli* β-galactosidase ($S_{20,w} = 16$ s), were co-sedimented with each heteroduplex and the distances from the meniscus normalized to a value of 30 s for the distance sedimented by the M13 DNA. This normalization yields values of 17 s for β-galactosidase, the secondary standard, and 34 to 35 s for the *N* activity of the heteroduplexes. These values are the same as those found in the previous experiment with homoduplexes and indicate that hetero- and homoduplexes sediment identically.

The *R* activity was also assayed throughout each gradient and its distribution found to be the same as that for *N*. In particular, no significant *R* activity was found at lower values of $S_{20,w}$, indicating the absence of active fragments containing *R* but not *N*. Fragments must contain one of the cohesive sites located at the ends of intact linear λ DNA to be active in our assay (Kaiser & Inman, 1965); consequently the activity of *R* can be detected in smaller fragments than that of *N* (Hogness *et al.*, 1966).

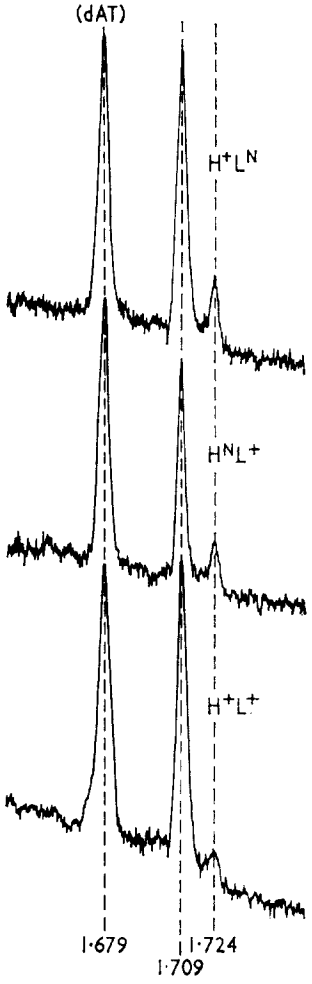


FIG. 2. Equilibrium sedimentation of the N_7 heteroduplexes and the wild-type homoduplex in neutral CsCl.

These hetero- and homoduplexes were formed from isolated single strands as described under Experimental Procedures section (b). The conditions of centrifugation and methods of calculation are given in the previous paper (Doerfler & Hogness, 1968) except that the initial density was somewhat lower ($1{\cdot}700 \pm 0{\cdot}001$ g ml.$^{-1}$).

(ii) *The* N*-activity of heteroduplexes in ultraviolet-irradiated cells*

The original incentive for the following experiments was based on the supposition that the *N* activity of the heteroduplex with mutant sequence in the template strand resulted from its conversion to the wild-type homoduplex by a system akin to that available for the repair of DNA containing ultraviolet photoproducts (Setlow & Carrier, 1964; Boyce & Howard-Flanders, 1964). Thus we supposed that the host cell contains, quite independently of λ, a system of enzymes which, on one hand, can recognize the mismatch of bases in a heteroduplex and eliminate it by excision of a segment of one strand, and, on the other hand, can fill in the excised region with new nucleotide subunits having a sequence directed by the unaffected strand (Hog-

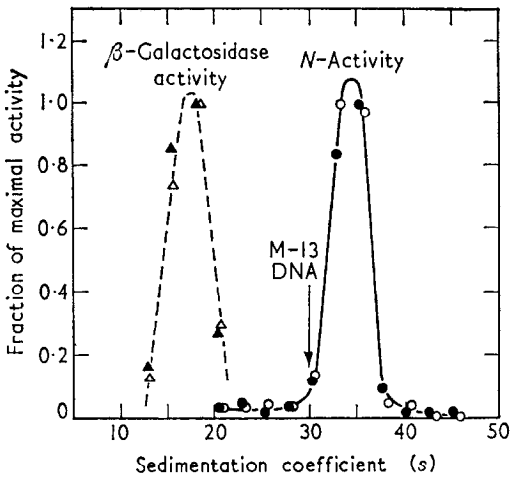


FIG. 3. Sedimentation coefficients of the active N_7 heteroduplexes.
The conditions of sedimentation and the method for calibrating the abscissa in $S_{20,w}$ values are given in the preceding paper (Fig. 10 of Doerfler & Hogness, 1968). The open symbols refer to the experiment in which H^+L^N was centrifuged; the filled symbols refer to the H^NL^+ centrifugation.

ness, 1966). The result would be the conversion of a heteroduplex to a homoduplex, either wild-type or mutant, depending upon which strand was excised. In its most general form, the supposition would include the formation of either homoduplex from either heteroduplex, resulting in the diminution of the *N*-activity of the inherently active heteroduplex by conversion to the mutant homoduplex, and increasing the activity of the other duplex from zero by conversion to the wild-type homoduplex. We shall return to these arguments in the Discussion; we allude to them here to clarify the rationale of the following experiments.

Irradiation of the host cells prior to infection with the helper phage should create photoproducts in the host DNA. If some of the enzymes used in the repair of ultraviolet-irradiated DNA are also necessary for the hetero- to homoduplex conversion, we may expect to trap an increasing fraction of such enzymes by increasing the exposure of the host cells to ultraviolet light. This should reduce the frequencies of conversion mentioned in the previous paragraph and thereby allow the differentiation of the two heteroduplexes.

The results of the experiment outlined above are given in Figure 4. The surviving fraction of *N* activity for the H^+L^+ homoduplex *versus* the dose of ultraviolet light applied to the host cells prior to helper infection is given in the upper part of the Figure. As would be expected, the ability of cells to become competent for wild-type DNA is much less affected by ultraviolet-irradiation than is cell viability, e.g. after 300 seconds of exposure, survival of colony formation is only 4×10^{-5}, while competence is little affected. The shift after 300 seconds to a more rapid rate of decay of competence remains to be explained.

Competence to detect *N* activity of the H^+L^N heteroduplex follows much the same decay curve as that for the H^+L^+ homoduplex (bottom curve of Fig. 4). If anything, it is less sensitive to ultraviolet irradiation than is competence for the homoduplex,

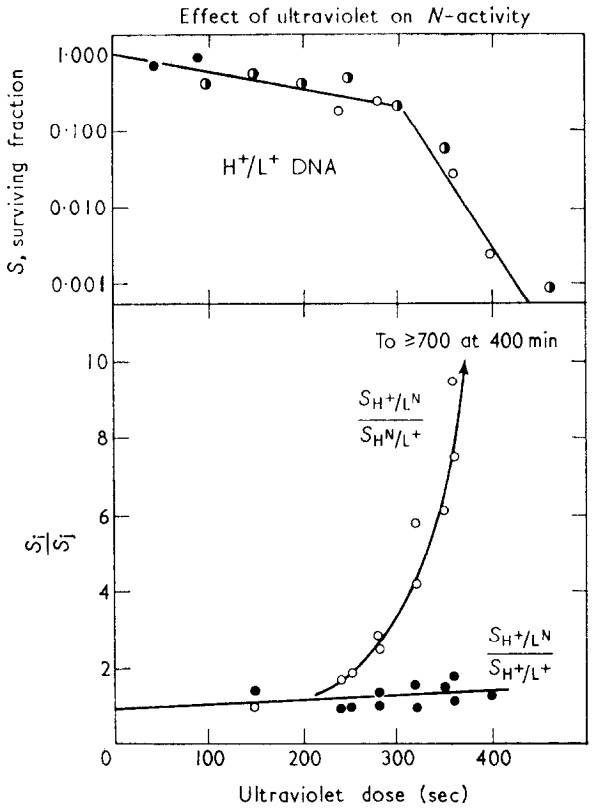


FIG. 4. Effect of ultraviolet irradiation of host cells on the N activity of the N_7 heteroduplexes. The ultraviolet irradiation of the cells (W3350), their subsequent infection with helper phage, λN_7, to render them competent, and the assay of the DNA are described under Experimental Procedures section (b). The different symbols in the top frame represent different experiments. In the bottom frame the ratio of surviving fractions of the two heteroduplexes ($H^+L^N : H^NL^+$, open symbols) and of the H^+L^N heteroduplex to H^+L^+ homoduplex (filled symbols) are plotted against dose, the same abscissa being used for both frames.

since the ratio of surviving fractions for H^+L^N to H^+L^+ tends to rise slightly. This is in keeping with the above arguments if H is the template strand for the N gene, since hetero- to homoduplex conversion provides a mechanism of inactivation of H^+L^N not applicable to the homoduplex. The loss of this conversion with irradiation could therefore lead to an increase in the H^+L^N activity relative to that of H^+L^+, the maximum increase being about twofold because of the twofold difference in activities observed in unirradiated cells (Table 1).

Of far more significance is the much greater sensitivity of competence toward the H^NL^+ heteroduplex (upper curve of the lower frame in Fig. 4). The difference in sensitivity is such that by 400 seconds of exposure, the H^+L^N heteroduplex is greater than 700-fold more active than the H^NL^+. Again, this is the expected result if H is the template strand for the N gene.

Two control experiments were performed which further substantiate this conclusion. The first is an experiment which parallels the previous one, the only difference being that the activity of R, rather than of N, was measured. No marked differences

in sensitivity of competence for *R* activity is expected among H^+L^+, H^+L^N and H^NL^+, since all three types of DNA contain the wild-type homoduplex structure in the *R* gene, and *N* activity is supplied by the $\lambda R_{54,60}$ helper phage used in the assay. The results are presented in Figure 5. They confirm the expectation and indicate that the differential effect of ultraviolet-irradiation of the host cells on the heteroduplex activities is limited to the *N* gene.

The results of the second control indicate that the difference in the *N* activities of the two heteroduplexes in irradiated cells is not due to a differential loss of the wild-type base sequence in the two heteroduplexes. In this control, the competent cells come from strain C600. These cells contain an amber suppressor (Weigert, Lanka & Garen, 1965), so that the amber mutants of *N* used as helper phage can supply the *N* function in the cells exposed to the heteroduplex molecules. (In previous experiments the competent cells were unsuppressed (W3350), and therefore the heteroduplex molecules had to supply the *N* function.) After helper infection and exposure to the heteroduplex DNA, these cells were then plated with unsuppressed indicating bacteria (W3350) to score for infective centers containing wild-type λ. Thus this method of assay simply asks for recovery of the wild-type base sequence of *N* in the initially infected cell, not for both the activity *and* the recovery of this gene.

Irradiation of the C600 cells prior to helper infection should not influence the relative activity of the two heteroduplexes in this assay. This is the result which was found. The H^+L^N and H^NL^+ heteroduplexes exhibit no significant difference in this assay for the *N* gene, whether or not the C600 were exposed to ultraviolet light over the range of doses shown in Figures 4 and 5.

(b) *The* $N_{7,53}$ *heteroduplexes, two positions of mismatch in the same gene*

Consider the case of a heteroduplex in which the template strand for *N* derives from the double mutant, $\lambda N_{7,53}$, and the complementary strand from wild-type, i.e. the $H^{NN}L^+$ heteroduplex, where NN stands for the sequence in the double mutant. If the postulated excision initiated near one position of mismatch has a low probability of including the second mismatch, then one would expect the conversion of $H^{NN}L^+$ to H^+L^+ to be a less probable event than the H^NL^+ to H^+L^+ conversion considered in the previous section. How much less probable would be determined by the probability of converting the mismatch at the N_{53} position to the wild-type structure and the degree of positive or negative interference between the two conversions.

The same type of argument may be invoked with regard to the fate of the H^+L^{NN} double heteroduplex, yielding the expectation that its *N* activity would be less than that of H^+L^N. Thus conversion to the mutant homoduplex structure at either of two positions, rather than just one, would inactivate this heteroduplex. Here the criteria for the degree of difference between the expected activities of the single and double heteroduplexes are the probabilities of these conversions prior to the DNA replication that should result from transcription–translation of *N*.

These considerations, and the desire to examine a completely non-leaky system in which the double mutant, $\lambda N_{7,53}$, is used both as helper phage and in the heteroduplexes, led to the following experiments. The strands of $\lambda N_{7,53}$ were isolated and individually coupled to the complementary wild-type strands to yield the H^+L^{NN} and $H^{NN}L^+$ heteroduplexes, using the same methods and conditions referred to in

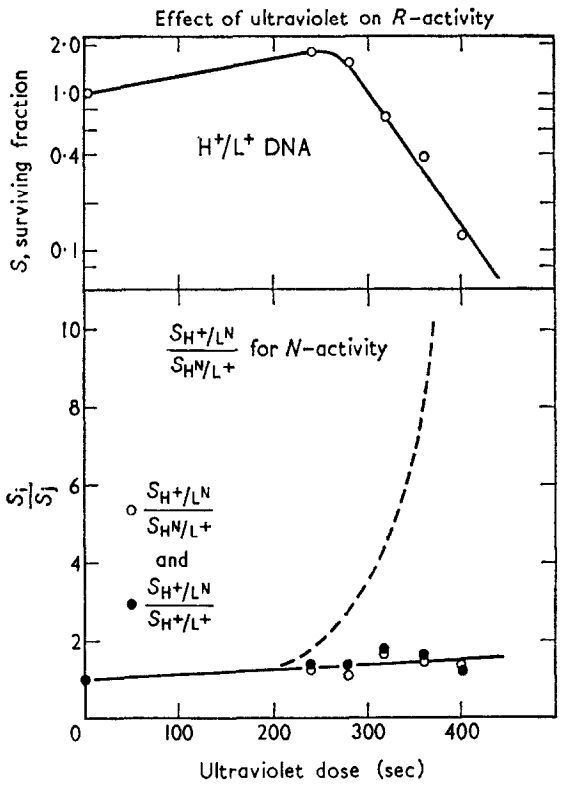


FIG. 5. Effect of ultraviolet irradiation of host cells on the R activity of the N_7 heteroduplexes. The conditions are the same as those given in Fig. 4, except that R activity was measured using $\lambda R_{54,60}$ as helper.

the previous section. Equilibrium sedimentation of each heteroduplex preparation in neutral CsCl yielded a band at a density of 1·710 g ml.$^{-1}$ and no material at the density of denatured DNA. The result is much like that given in Figure 2, except that the buoyant densities of the double heteroduplexes appeared to be 0·0009 g ml.$^{-1}$ higher than those observed for the single heteroduplexes. We cannot be sure that this small difference is statistically significant.

The activities of the double heteroduplexes are given in Table 2. Because of the low R activity in the first preparation of heteroduplexes (about one-fifth of the specific activity of H^+L^+, Table 2), a second preparation was made. When sedimented to equilibrium in neutral CsCl, the distributions given by these second preparations were essentially the same as those given by the first. The activities are given in Table 2. While the R activities are higher (about two-thirds the specific activity of H^+L^+), the N activities are lower. This difference calls into question the use of N- to R-activity ratios to account for different efficiencies of renaturation, a subject we shall return to in the discussion.

However, whether one compares the specific activities of N or the ratio of N to R activity in either preparation, the general conclusions are the same. Both double heteroduplexes exhibit a significantly lower N activity than do the single heteroduplexes, and the H^+L^{NN} heteroduplex has a twofold higher N activity than the

$H^{NN}L^{+}$ heteroduplex, a difference not found for the *R* activities. These results are in qualitative agreement with the expectations given above.

The effect of ultraviolet irradiation on the competence of unsuppressed host cells was also determined for the double heteroduplexes and is shown in Figure 6. The *N* activity of $H^{+}L^{NN}$ relative to that of $H^{NN}L^{+}$ increases from 2 in the unirradiated

TABLE 2

Activity of the $N_{7,53}$ *heteroduplexes*

DNA†	Preparation 1		Preparation 2	
	Specific activity of *N*‡ (normalized to $H^{+}L^{+}$) (%)	*N* activity/*R* activity‡ (normalized to $H^{+}L^{+}$) (%)	Specific activity of *N*‡ (normalized to $H^{+}L^{+}$) (%)	*N* activity/*R* activity‡ (normalized to $H^{+}L^{+}$) (%)
$H^{+}L^{+}$	100	100	100	100
$H^{NN}L^{NN}$	<0·05	<0·05	—	—
$H^{+}L^{NN}$	4·5	20	1·4	2·1
$H^{NN}L^{+}$	2·2	11	0·9	1·3

† Two preparations of the heteroduplexes and the $H^{+}L^{+}$ homoduplex were prepared as described in Table 1 and the text; the $H^{NN}L^{NN}$ homoduplex was included in only one of these.

‡ Activities were measured as in Table 1, except that $\lambda N_{7,53}$ was used as helper in the *N* assay. The values are the average of two independent assays (duplicate plates being registered in each assay), with range $< \pm 35\%$ of the average.

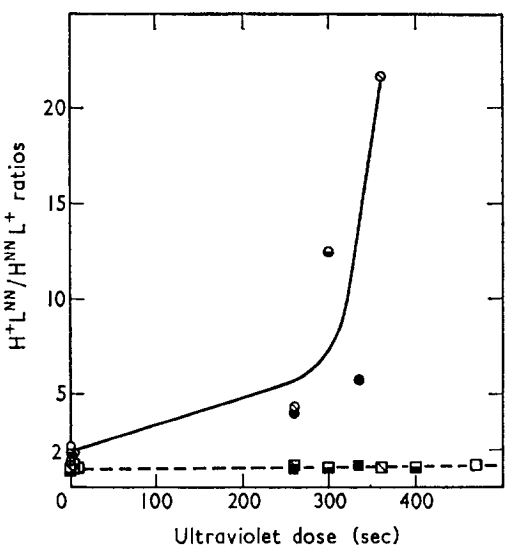


FIG. 6. Effect of ultraviolet irradiation of host cells on the *N* and *R* activities of the $N_{7,53}$ double heteroduplexes.

The conditions are the same as those given in Figs 4 and 5, except that $\lambda N_{7,53}$ was used as helper phage in the assay of *N* activities. The circles represent the ratios of *N* activities, and the squares the ratios of *R* activities. The five different types of circles and squares represent five independent experiments.

———, *N* activities; – – – –, *R* activities.

cells to greater than 20 after 360 seconds exposure, the observance of higher values at larger doses being restricted by the lowered *N* activity of the double heteroduplexes. As occurred with the single homoduplexes, the ratio of *R* activities changes very little over the entire range of doses.

(c) *The O_{29} heteroduplexes*

The results with the *N* heteroduplexes encouraged us to extend this approach to *O*. The two heteroduplexes, H^+L^o and H^oL^+, were constructed using the amber mutant, λO_{29}, as the source of the mutant strands. The equilibrium distribution of each preparation when sedimented in neutral CsCl consisted of only one band, its maximum being at 1·710 g ml.$^{-1}$ and exhibiting the slight asymmetry toward higher densities found for all heteroduplexes and renatured homoduplexes we have examined.

The *O* activity of these heteroduplexes is given in Table 3. There is no significant difference in the two heteroduplex activities, whether the specific activities of *O* or the ratio of *O* to *R* activities is used as the basis for comparison.

TABLE 3

Activity of the O_{29} heteroduplexes

DNA†	Specific activity of *O*‡ (normalized to H^+L^+) (%)	*O* activity/*R* activity‡ (normalized to H^+L^+) (%)
H^+L^+	100	100
H^+L^o	83	138
H^oL^+	87	168

† The different DNA's were prepared as described in Table 1.

‡ Activities were measured as in Table 1, except that λO_{29} was used as helper in the *O* assay. The values are the average of two independent assays with range $< \pm 10\%$ of the average.

When the unsuppressed host cells were irradiated prior to infection with helper phage (as in Figs 4, 5 and 6), the change in the H^+L^o to H^oL^+ ratio for *O* activity is the same as that for *R* activity, the two curves looking very much like that for the *R* activity of the N_7 heteroduplexes given in Figure 5. Doses up to 520 seconds were examined. Thus the method fails to distinguish the template strand for the *O* gene.

4. Discussion

(a) *Orientation*

The conclusion drawn from the experiments with the heteroduplexes of *N* is that the template strand for this gene is H, the strand with 5′-to-3′ direction running from left to right in Figure 1. Hence, transcription of *N* proceeds from right to left.

This conclusion is based on the assumption that transcription of *N* in heteroduplexes does occur in the irradiated host. The essentially qualitative difference that exists in the *N* activities of the two heteroduplexes, with H^+L^N being active and H^NL^+ inactive, is interpreted as indicating the quality of the mRNA formed by each.

This interpretation is supported by the results of the two control experiments, which indicate that no differential destruction of one heteroduplex molecule over the other is caused by the irradiation. The assumption is also supported by the equivalence of the N activities of the H^+L^+ homoduplex and the H^+L^N heteroduplex in highly irradiated cells, a result consistent with the interpretation that the activity of H^+L^N derives from its direct transcription, as must be the case for H^+L^+.

The argument defining H as the template strand can thus be separated from a consideration of the molecular mechanism by which the H^NL^+ heteroduplex yields N activity in non-irradiated cells. Whatever the mechanism, it is lost by irradiation of the cells, leaving, we assume, only the mechanism of direct transcription of N in the heteroduplex. Before considering the relevance of our results to a definition of this mechanism, we find it convenient to relate the orientation of N to the other data concerning gene orientation in λ.

Heteroimmune infection experiments indicate that the N function is directly controlled by the immunity repressor (B. Egan, personnel communication; Thomas, 1966), the structural gene of which is C_I (Ptashne, 1967*a*). The orientation of N dictates that a target for this repressor be located to the right of this gene (or at its right-hand end) and to the left of C_I (Fig. 1). It is known that the C_I repressor reacts specifically with the DNA in the λ immunity region, i^λ (Ptashne, 1967*b*). Thus it forms a complex with λ DNA but does not react with the DNA from the hybrid, λi^{434}. This hybrid is non-homologous with λ only over the segment extending from t_{27} to x (inclusive), but not including N_7 and other amber mutations of N (Eisen *et al.*, 1966). Since the defective mutant, t_{27}, does not complement amber mutants of N, and lies within i^λ between mutations in N and those in C_I, it is reasonable to suppose that the mutation affects the interaction of repressor and its target (operator).

The situation is analogous to defective mutations in x (e.g. t_{11}) which do not complement C_{II} or O, two genes which also appear to be under the direct control of the C_I-repressor (Bode & Kaiser, 1965; Pereira da Silva & Jacob, 1967). Unfortunately, our experiments failed to define the orientation of O, for reasons we discuss in the final section. However, one would suppose that the repressor target of the C_{II}–O operon is located within i^λ and therefore that the orientation of this operon be opposite to N. Consequently, we may suppose that the t_{11} type mutants in x affect the interaction between the repressor and this target.

We do not yet know the extent of the operon that includes N. The results of Bode & Kaiser (1965) indicate that the C_{III} function could not be induced by superinfecting heteroimmune phage. Thus we suppose that C_{III}, like N, is under direct control of the C_I-repressor, which would be consistent with its inclusion in the N operon. A few genes located to the left of C_{III} and to the right of b_2 (Fig. 1) have recently been uncovered. These include genes for: (1) the λ exonuclease and β-proteins (Radding, Szpirer & Thomas, 1967); (2) the curing and integration of prophage (Zissler, 1967; Franklin, 1967; Yarmolinsky, 1967); and (3) recombination between λ genomes (Franklin, 1967; H. Echols, personal communication; E. Signer & J. Weil, personal communication). Hybridization experiments with RNA formed after induction of lysogens carrying λt_{11} (Taylor *et al.*, 1967; Cohen & Hurwitz, 1967) have given results which indirectly indicate that the orientation of these genes is the same as N, from right to left. When separately combined with each λ strand, this λ-specific RNA formed a hybrid almost exclusively with the strand we term H. Although λt_{11} is defective in the expression of late genes (Protass & Korn, 1966), it allows the synthesis

of abnormally large amounts of λ exonuclease and β-protein (Radding & Shreffler, 1966), and cures with exceptionally high frequencies (Eisen *et al.*, 1966). It is reasonable to suppose that the RNA examined is transcribed from these genes and hence that the genes between C_I and b_2 have the same orientation. This makes it possible to imagine that the N operon extends to the left to include one or more of these genes. One might hope to determine the amount of that extension by measuring the size of the RNA which contains the N sequence. While there is indirect evidence that the RNA transcribed from the N–b_2 region includes molecules containing as many as 5000 bases (Kourilsky & Luzzati, 1967; Kourilsky, Luzzati & Gros, 1967), the molecules containing the N sequence have not yet been identified.

Another approach for determining the extent of the N operon is to examine the effect of mutations in N on the activity of genes to its left. Amber mutations in N severely reduce the amounts of λ exonuclease and β-protein (Radding & Shreffler, 1966), and exhibit decreased ability to cure (Eisen *et al.*, 1966; Franklin, 1967). The effect of such ambers could be attributed to polarity within a single operon. Alternatively, the protein product of N may be necessary for the normal expression of these genes. Recent evidence that temperature-sensitive mutants of N produce little, if any, active λ exonuclease at the non-permissive temperature (A. D. Kaiser, personal communication) tends to enforce the latter alternative. Hence the existing data regarding the effects of mutations in N do not yet form a basis for an argument that the N operon includes genes lying between N and b_2.

Whether or not there are other regions in λ which are oriented from right to left is not known. The region to the left of b_2 appears to be uniquely oriented in the opposite direction, as is a very significant fraction of the total region to the right of b_2 (Taylor *et al.*, 1967; Cohen & Hurwitz, 1967). As was indicated in the previous paper (Doerfler & Hogness, 1968), this distribution of template activity for transcription between the strands parallels the distribution of GT content between strands. In the left arm, the GT content of L is much less than that of H, and L appears to be the only strand used as template; in the right arm, the GT contents of H and L are the same, and both are used as templates. Thus it may be that a GT content less than 0·50 characterizes the strand used as template in a segment of λ DNA. Indeed, this may not be unique to λ, since Guild & Robison (1963) found indirect evidence indicating that the strand of pneumococcal DNA with lower buoyant density in alkaline CsCl was used as template in the transcription of genes required for resistance to certain antibiotics. We refer the reader to the preceding paper (Doerfler & Hogness, 1968) for a further consideration of this correlation as well as that involving the binding sites for poly rG.

(b) *Heteroduplex to homoduplex conversion*

We have already considered most of the consistencies between our results and a general model of excision and repair in outlining the rationale for each experiment. Here we entertain certain extensions of this model for heteroduplex to homoduplex conversion.

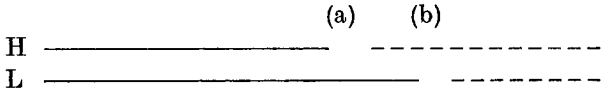
Were excision and repair the dominant reaction encountered by infecting heteroduplexes of all types, and were each strand of any given heteroduplex excised with equal probability, then each member of a reciprocal pair of single heteroduplexes should exhibit an activity that is about 50% that of the wild-type homoduplex. The heteroduplex with wild-type sequence in the template strand might be some-

what higher due to escape from excision and repair *via* replication after transcription–translation of the requisite genes. The reciprocal heteroduplex should not exceed the 50% value. It should be noted that the λ DNA infection takes place over a 30-minute period in an environment (0·01 M in Tris–HCl, $CaCl_2$ and $MgSO_4$, pH 7) devoid of sources for energy, carbon, phosphate and nitrogen. In this condition, excision and repair may occur whereas replication, with its prerequisite of extensive transcription–translation, does not.

On the same assumptions, double heteroduplexes should have about 25% the activity of the wild-type homoduplex, providing the two heteroduplex regions behave independently. Considering all three types of heteroduplexes tested (N_7, $N_{7,53}$ and O_{29}), only the N_7 exhibits activities close to those predicted on the above assumptions of equivalence. The average values for the $N_{7,53}$ are lower than the 25% predicted; the O_{29} values are higher than 50%.

These differences can be accommodated under a model of excision and repair if it can be supposed that the three reciprocal pairs of mismatched bases ($+N_7$, N_7+; $+N_{53}$, $N_{53}+$; $+O_{29}$, $O_{29}+$) are different. There are eight possible reciprocal pairs of mismatches in which one strand is wild type and the other amber (H^+L^{amber}, $H^{amber}\ L^+$ = GT, AC; TT, AA; CT, AG; AA, TT; GA, TC; CA, TG; AG, CT; or GG, CC), the ochre–amber heteroduplexes (TG, CA) being eliminated. The amber mutants used here were isolated by Campbell (1961) after ultraviolet restoration (Weigle, 1953) in a strain containing the amber suppressor, *su*-2^+, which can translate the amber codon as glutamine (Weigert *et al.*, 1965). The nature of this mutagenic agent does not allow elimination of any of the remaining six transversions and two transitions which yield ambers. We might consider the transversion from lysine or from glutamate as unlikely, since the substituted amino acid in the suppressed protein would differ in charge from that in the wild type. However, this would still leave six allowable reciprocal pairs of heteroduplex structures, which is more than sufficient.

Consideration of a heteroduplex-to-homoduplex conversion involving excision and repair need not be restricted simply to the elimination of the mismatch; it may, for example, result from recombination between heteroduplex and helper phage DNA. It has been suggested (Meselson, 1964) that an early intermediate in λ recombination consists of parental fragments joined by single-strand extensions containing overlapping sequences:



If the heteroduplex fragment is represented by the solid line with the mismatch originally in position (a), then one type of homoduplex would be generated by repair synthesis in that region; on the other hand, were the heteroduplex fragment represented by the dashed line with the mismatch in position (b), the other type of homoduplex would be generated. Thus a given heteroduplex could be converted to either homoduplex structure by the same mechanism of recombination.

The results of the experiments in which the host cells were irradiated with ultraviolet light prior to infection with the *N* heteroduplexes do not necessarily need re-interpretation to fit this recombination model, since there is evidence that genetic recombination and the repair of ultraviolet-induced photoproducts may involve some common steps (Clark & Margulies, 1965; Howard-Flanders & Boyce, 1966).

If recombination with helper DNA were the mechanism whereby the H^NL^+ heteroduplex exhibited activity in non-irradiated cells, then one would suppose it to be the dominant reaction peculiar to infecting heteroduplexes. Thus in the assay for the *R* activity of the various *N* and *O* heteroduplexes, it may be that they recombine with the helper phage at the heteroduplex position. Depending upon which parental fragment in the recombinant comes from the heteroduplex, the R^+ gene in the heteroduplex may be recovered or lost. On this basis, one might expect the *R* activity in the heteroduplexes to be less than that of the homoduplexes. In the experiments referred to in Tables 1, 2 and 3, the average *R* activity of the N_7, $N_{7,53}$ and O_{29} heteroduplexes was 76, 42 and 57% the activity of the renatured homoduplexes, the members of a given reciprocal pair of heteroduplexes exhibiting equivalent *R* activities.

This extension of the excision and repair model is also useful in suggesting an explanation for the lack of a differential effect of ultraviolet light on the O_{29} heteroduplexes. While irradiation of the host cells can be imagined to trap enzymes of the host that are required for the excision and repair of recombination, it is unlikely that this irradiation will make unavailable enzymes supplied by transcription–translation of the infecting λ DNA. As was mentioned previously, the λ genes specifying such enzymes are located to the left of C_{III}. Although conclusive evidence regarding their control is not yet available, it seems probable that the rate of expression of these genes is reduced in amber mutants of *N*. There is, on the other hand, no reason to suppose that their expression is restricted in *O* mutants; e.g. the λ exonuclease and β-protein are made in at least normal amounts by *O* mutants (Radding & Shreffler, 1966). Infection of irradiated bacteria with the λO_{29} helper phage could result in the synthesis of those components necessary for heteroduplex-to-homoduplex conversion and which are not available in irradiated cells, thus accounting for the activities of the O_{29} heteroduplexes in such cells.

These ideas make relevant a test of the *N* heteroduplex activities in host cells which are deficient in recombination (*rec*) or in reactivation of ultraviolet-irradiated phage (*uvr*), both types of mutants being partially deficient in repair of DNA containing ultraviolet-photoproducts (Howard-Flanders & Boyce, 1966). We have examined one *rec* mutant and one mutant in each of the three *uvr* loci† without observing a striking effect on the ratio of *N* activities for the N_7 heteroduplexes, using $\lambda N_{7,53}$ as helper. In the case of the *rec* mutant, we observed a consistent two-fold increase in the H^+L^N/H^NL^+ activity ratio over that found using the rec^+ strain from which the mutant was derived. In the other mutants, no significant differences were observed. While the effect of *rec-13*, the recombination-deficient mutant, is in the right direction, it is of marginal amplitude. The lack of a striking effect may result from a low order of suppressor activity in all of the strains, except the *uvrA-6* mutant. Thus the *rec-13*, *uvrB-5* and *uvrC-34* derive directly from a strain which, while like its derivative mutants, is non-permissive toward $\lambda N_{7,53}$ allows N_7 and N_{53} to form quite small plaques with an efficiency of about 0·5 relative to C600. We find this strain to be non-permissive with respect to $\lambda R_{54,60}$ and $\lambda N_7 R_{60}$, and N. Franklin (personal communication) found the same result with two amber

† The *rec* mutation is *rec-13* in strain AB2463 (Howard-Flanders & Theriot, 1966) and the *uvr* mutations are *uvrA-6* in AB3042, *uvrB-5* in AB1885 and *uvrC-34* in AB1884 (Howard-Flanders, Boyce & Theriot, 1966). These strains exhibit the following efficiencies of plating for $\lambda N_{7,53}$: 4×10^{-7}, 1×10^{-6}, 3×10^{-5} and 3×10^{-5}, respectively.

mutants of T4 phage. While the significance of this heterogeneity of suppression is not clear, the fact that single mutants of *N* can form plaques indicates that mutant RNA transcribed from H^NL^+ may yield active *N* product and thus reduce the effect of these *rec* or *uvr* mutations on the H^+L^N/H^NL^+ activity ratio. This argument probably does not apply to the strain containing *uvrA-6*, since in this case the λN_7 form plaques with an efficiency of only 1×10^{-5}.

We conclude by indicating some of the considerations that have led us to de-emphasize other possible models. The results obtained when using the double mutant, $\lambda N_{7,53}$, either as helper or in heteroduplex, reduce the effectiveness of the argument that the leakiness in the helper allows expression of *N* activity with H^NL^+. Furthermore, such leakiness would have to be sensitive to ultraviolet-irradiation of the host.

We consider unlikely, though not eliminated, models involving semi-conservative replication of the infecting heteroduplex. The objection is not that some component of the system for this replication must be provided by the host and be made unavailable by ultraviolet irradiation; such a proposition is quite reasonable. Rather, we think that the lower activity of both $N_{7,53}$ heteroduplexes relative to the N_7 heteroduplexes is difficult to explain solely by a replication model, without the addition of *ad hoc* reactions specific to heteroduplexes and not relevant to their replication. *A priori*, there appears no reason why the replication of a double heteroduplex should not proceed as efficiently as the replication of a single heteroduplex.

In addition, we have carried out assays for the *N* activity of N_7 heteroduplexes in the presence of inhibitors of DNA replication (nalidixic acid and 5-fluorodeoxyuridine) without observing any change in their relative activity, even at the high inhibitor concentrations required to inhibit the *N* activity of native λ DNA by 95 to 99%. However, we cannot use this result to eliminate the possibility of the critical first round of replication, since we have not determined the amount of λ DNA synthesized under such conditions.

In summary, we offer the general excision and repair model as the most effective explanation for the activities exhibited by heteroduplexes under our conditions of assay. We suggest that recombination may be the specific mode for effecting the excision and repair that result in the conversion of heteroduplex structures to either of their homoduplex analogs.

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