Analysis of *nutR:* A Region of Phage Lambda Required for Antitermination of Transcription

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Summary

The *N* gene product of coliphage lambda acts with host factors (Nus) through sites (*nut*) to render subsequent downstream transcription resistant to a variety of termination signals. These sites, *nutR* and *nutL,* are downstream, respectively, from the early promoters P_R and P_L. Thus a complicated set of molecular interactions are likely to occur at the *nut* sites. We have selected mutations in the *nutR* region that reduce the effectiveness of pN in altering transcription initiating at the P_R promoter. DNA sequence analysis of three independently selected mutations revealed, in each case, a deletion of a single base pair in the *cro* gene. Consideration of the effect of such mutations on the extension of translation of *cro* message into the adjacent downstream *nut* region led to the identification of a consensus sequence CGCTCT(T)TAA that appears to play a role in the recognition of a host factor, possibly the NusA protein.

Introduction

The *N* gene function (pN) of coliphage lambda regulates phage gene expression by permitting transcription to overcome termination signals (Roberts, 1969). This mode of regulation is shared by the family of lambdoid phages. These phages (for example, coliphages 434 and 21 as well as Salmonella phage P22) have genes located in the same relative position as the lambda *N* gene (Figure 1) that code for *N*-like functions (Friedman et al., 1973; Hilliker and Botstein, 1976). Although similar in action, these functions are specific: they act only to influence gene expression from their homologous DNAs (Thomas, 1966; Dambly et al., 1968; Couturier and Dambly, 1970; Herskowitz and Signer, 1970). One exception to this exclusivity are lambda and 434, which share the same *N* gene (Thomas, 1966).

Host-determined functions and unique sites on the phage DNA are necessary both for transcription termination and for pN-induced antitermination. The host factors include RNA polymerase (Ghysen and Pironio, 1972; Georgopolous, 1971b; Sternberg, 1976; Baumann and Friedman, 1976), Rho transcription termination factor (Roberts, 1969; Brunel and Davison, 1975; Das et al., 1976; Inoko and Imai, 1976; Richardson et al., 1977) and a series of additional host factors, called Nus (Georgopoulos, 1971a; Friedman,

1971; Friedman and Baron, 1974; Keppel et al., 1974; Friedman et al., 1976, 1981; Greenblatt et al., 1980). The sites include putative sites of pN action, called *nut* (Salstrom and Szybalski, 1978; Rosenberg et al., 1978), as well as termination signals (Roberts, 1969; Rosenberg et al., 1978; Rosenberg and Court, 1979).

Consideration of the nature of the various factors involved in pN action formed the basis for the following model of pN action (discussed by Friedman and Gottesman, 1982). Transcription initiating at the early promoters P_R and P_L extends through the *nutR* and *nutL* sites, respectively (Figure 1). At these sites RNA polymerase is modified, rendering continuing transcription resistant to termination signals. The nature of the promoter appears to play no role in pN action, since placement of the *nutR* region downstream from the bacterial *gal* operon promoter results in termination-resistant transcription in the presence of pN (de Crombrugghe et al., 1979). Analysis of the *nut* region was facilitated by the isolation of mutations that interfere with pN modification of transcription initiating at P_L (Salstrom and Szybalski, 1978). These mutations (*nutL*⁻) map between P_L and the *N* gene (Figure 1). Sequence analysis located the mutations immediately downstream from P_L in the unpaired center of a region of hyphenated dyad symmetry (Rosenberg et al., 1978). The identification of a similar sequence (16 of 17 bp) downstream from P_R, distal to the *cro* gene, supported the argument that this sequence of dyad symmetry was involved in pN action (Rosenberg et al., 1978). Although these studies strongly implicate the 17 bp sequence in pN action, we raise two unanswered questions concerning the nature of the *nut* site: Is the 17 bp sequence sufficient for full pN activity? and, What is the nature of the interactions at *nut* sites?

We report the isolation of mutations downstream from P_R that influence the ability of pN to modify transcription initiating at P_R. Furthermore, we focus attention on *nutR62,* a representative of the class of mutations that is not located in the core 17 bp *nut* sequence. The location of *nutR62* upstream of the core sequence suggests that additional sequences may be necessary for full activity of pN. Consistent with the argument of an extended *nut* region is our finding of a sequence upstream from the core *nut* sequence common to lambda *nutR* and *nutL* regions as well as the analogous regions of phages 21 and P22. To simplify our discussion and to have a consistent nomenclature, we will refer to the 17 bp sequence of dyad symmetry as the core sequence, and to the extended sequence from the 3' end of *cro* to t_{R1}, including the core, as the nut region.

Results

Selection and Initial Mapping of *nutR* Mutations
The strategy used in the selection was based on the phenotype expected for *nutR* mutations; initiation of

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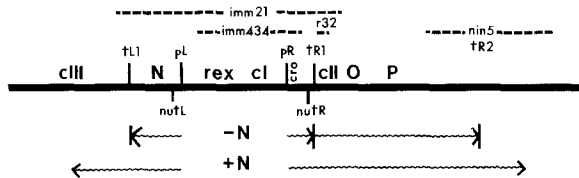
Figure 1. Arrangement of Genes and Regulatory Elements in the Region from 69% to 90% of the Lambda Genetic Map

Dashed lines: extents and locations of the *imm434* and *imm21* substitutions, the *r32* insertion and the *nin5* deletion. Wavy lines: transcripts from P_R and P_L under both N$^+$ and N$^-$ conditions.
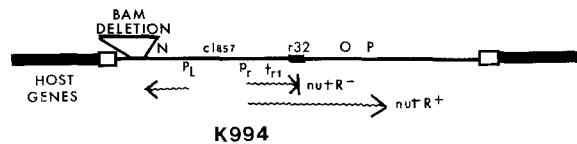

Figure 2. Lysogen Used for Selection of *nutR* Mutants

Relevant mutations used in the *nutR*-selecting strain are Bam HI deletion, *cl857* and *r32*. Upon thermal induction, transcription from P_R in a *nut*$^+$ prophage will proceed through the *r32*. Replication functions (*O* and *P*) will be expressed, and the cell will die because of in situ replication (Eisen et al., 1968; Calef and Neubauer, 1968). Cells that survived induction were screened for NutR$^-$ phenotype, as described in the text. Throughout this study all phages with *nutR* mutations also have the *cl857* mutation.

transcription from P_R should be unaffected, but subsequent transcription in the presence of pN should not be termination-resistant. The selection employed a lysogen containing a lambda variant with three relevant changes from wild-type (Figure 2). The first was a temperature-sensitive *cl* mutation (*cl857*) that results in the synthesis of a thermolabile repressor. The prophage can be induced by shifting the lysogens to temperatures above 38°C (Sussman and Jacob, 1962). The second was a deletion of genetic material in the P_L operon between two Bam HI restriction sites (Kourilsky et al., 1978) that eliminates functions unessential for lytic growth, but lethal to the host (Greer, 1975). A prophage with the Bam HI deletion also fails to excise from the bacterial chromosome because it is Int$^-$ (Gottesman and Yarmolinsky, 1968). The third was an IS2 element in the *cll* gene, *r32*, that is polar, causing the expression of replication functions to be absolutely dependent on the pN stimulation of P_R-promoted transcription (Brachet et al., 1970; Tomich and Friedman, 1977). When this lysogen is thermally induced, expression of replication functions is lethal to the cell (Eisen et al., 1968). The lysogen carrying the prophage with the above characteristics is K994.

Possible candidates for *nutR* mutations were obtained as survivors of growth of K994 at 40°C. A variety of different mutations in addition to *nutR*$^-$ would be expected to permit survival of the lysogens (for example, mutations in the prophage, such as in genes or sites involved in replication (*O*, *P* or *ori*), in the *N* gene or in the P_R promoter (Eisen et al., 1968; Calef and Neubauer, 1968), as well as mutations in bacterial genes, such as *nus* (Friedman et al., 1981). However, *nutR* mutations should exhibit unique phenotypic traits: genes in the P_L operon, for example, *N*, should be expressed; the *cro* gene, which precedes t_{R1} and IS2, should be expressed; and functions distal to t_{R1} and IS2 should not be expressed, even in the presence of pN. Moreover, even though they are not expressed, replication genes should be present in an intact form. Lysogens were screened by complementation and recombination tests (see Experimental Procedures) to determine if they met these criteria. Four of eighty survivors were identified as having prophages with potential *nut* mutations.

Complementation tests showed that when these lysogens were induced they supported growth of

$\lambda cro27$, λN *am7,53* and λP *am3*, but not growth of λO *am29*. However, recombination tests showed that the lysogens had intact *O* genes, since λO *am29* formed O$^+$ recombinants in these lysogens at about the same frequency as it formed them in an O$^+$ control lysogen. Since *P* product is required at low levels (Thomas, 1966; Thomas et al., 1967), the finding that putative *nutR* mutants can express complementing levels of P is not inconsistent with a reduction in transcription past the *r32* insertion.

Genetic crosses were used to map four putative *nutR* mutations in relation to the *r32* insertion (data not shown). In all four mutants (*nutR37, nutR57, nutR62* and *nutR64*) the *nutR* mutations are closely linked to the *r32* insertion. The *nutR*$^-$ phages were isolated from their respective lysogens by the use of a host deficient in Rho-dependent termination activity (Das et al., 1976) in the following manner. Each of the four lysogens were infected with $\lambda imm434$, and resulting progeny were plated on K1086, a 434 lysogen of the *rho ts15* strain K1024, to isolate lambda recombinants. Phages isolated in this way grew in the Rho-deficient strain at 35°C, but not in a Rho$^+$ host. Since $\lambda r32$ grows in the *rho*$^+$ host under the same conditions, these mutant phages were assumed to have defects in antitermination of transcription initiating at P_R.

Complementation studies with lysogens and free phages yielded a more precise mapping of the putative *nutR* mutations. Using the procedures discussed below, we were able to locate all of the putative *nutR* mutations to the right of P_R and to the left of t_{R1}. Since all of these mutations mapped in an identical manner, we will discuss their mapping as one class.

The complementation studies used in the initial screening suggest that the *nutR* mutations are located to the right of P_R. If the putative *nutR* mutations are located in or to the left of P_R, one of three possible changes might be expected to result in a NutR phenotype. First, the mutations might eliminate P_R as an active promoter. This seems unlikely, since prophages with the *nutR* mutations express Cro. Second, the mutations could result in the expression of a P_L-encoded function that acts in trans to interfere with

expression of P_R-encoded functions distal to t_{R1}. This also seems unlikely, since the nutR mutations are cis-acting. Third, the mutations might eliminate the action or expression of a function that normally acts to stimulate transcription of genes in the P_R operon distal to t_{R1}. Two facts argue against this: first, the Bam HI deletion in the selecting prophage eliminates most genes in the P_L operon; second, an infecting lambda with the P_L operon intact fails to stimulate O gene expression from the nutR⁻ prophages.

The nutR mutations were located to the left of t_{R1} by recombination studies with a phage that has a substitution of genes from the related phage 434 in the cl–P_R region, called imm434 (Kaiser and Jacob, 1957), and carries a mutation in t_{R1}, called cin1 (Wulff, 1976). The imm434 substitution includes the cro and cl genes, and is easily identified because phages carrying this substitution can grow in a lambda lysogen. Details of the cross are outlined in the legend to Figure 3. In brief, when a λimm434 cin1 is crossed with a λnutR⁻ r32, only recombinants that have wild-type alleles for the nutR and cin1 mutations as well as the IS2 insertion should be able to form a plaque on a host with two mutations, nusA1 (Tomich and Friedman, 1977) and himA42 (Miller and Friedman, 1980). As shown in Figure 3, if a nutR mutation is located to the left of the cin1 mutation, then the most frequent class of recombinants that grow on the double mutant should be those with the imm434 substitution. We find this to be the case for each of the nutR mutations.

Effect of Rho on Growth of λnutR⁻

At 35°C λnutR⁻ r32 phages grow poorly in Rho⁺ hosts that support the growth of the λr32 parental

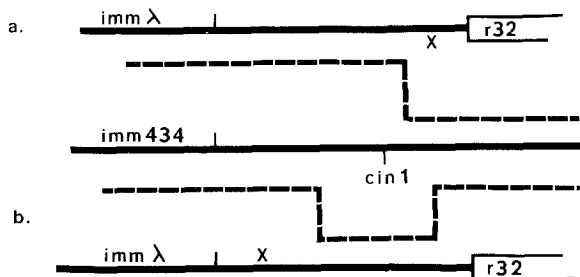Figure 3. Mapping nutR Mutations with Respect to t_{R1}

λr32 derivatives with putative nutR mutations (X), nutR37, nutR57, nutR62 and nutR64, were crossed with λimm434 cl cin1. The cin1 mutation is a change of a single base pair at the beginning of t_{R1} (Rosenberg et al., 1978). Phages with the cin1 mutation will not form plaques on a himA host (Williams et al., 1977). Neither phages with the r32–IS2 insertion (Tomich and Friedman, 1977) nor nutR mutations (see text) will form plaques on a nusA host. Recombinants from the aforementioned cross that are cin⁺ nutR⁺ can therefore be selected for on a himA–nusA double mutant at 32°C. The nutR mutations can be mapped as follows. (a) If the nutR mutation lies to the right of cin1, the majority of cin⁺nutR⁺ recombinants should have lambda immunity. (b) If the nutR mutation lies to the left of cin1, the majority of cin⁺nutR⁺ recombinants should have 434 immunity. Fifty plaques from each cross were checked, and all had 434 immunity.

phages. The fact that λnutR⁻ r32 phages grow as well as the λr32 parent in hosts with the rho ts15 mutation at 35°C suggests that the defect in the nutR⁻ mutants reduces the ability of the phages to overcome Rho-determined transcription termination; N modification is incomplete. Consistent with this argument is the fact that transcription termination both at t_{R1} (Roberts, 1969; Rosenberg et al., 1978) and within the r32–IS2 insertion depends on Rho action (de Crombrugghe et al., 1973).

To study the effect of nutR⁻ in a more quantitative manner, we looked at one nutR mutant, nutR62, in single-step growth experiments. The effect of the nutR62 mutation was studied both in the presence and in the absence of the r32–IS2 element. We looked first at experiments with λnutR62 r32. Because the IS2 element contains a strong Rho-dependent termination signal, growth of this phage offers a very sensitive assay of pN action. As shown in Table 1, λr32 grows in the Rho⁺ host at all temperatures. However, the nutR62 r32 derivative grows poorly in the Rho⁺ host at 35°C and 40°C, even though it grows well in the Rho⁻ host at 35°C. This suggests that the failure to grow results from an inability of the nutR62 derivative to overcome Rho-dependent termination. Second, we looked at experiments with nutR62 derivatives without the IS2. As shown in the single-step growth curves in Figure 4, this phage grows poorly in Rho⁺ hosts at higher temperatures, in this case 42°C. That this failure to grow is due to a failure of pN modification of transcription initiating at P_R was shown by experiments with a nutR62 derivative carrying the nin5 deletion. The nin5 deletion (see Figure 1) removes a strong terminator (t_{R2}) in the P_R operon, resulting in N-independent phage growth (Court and Sato, 1969). Thus the removal of the t_{R2} termination signal reduces the need for N modification of transcription at nutR. As shown in Figure 4, the growth pattern of λnutR62 nin5 in a Rho⁺ host at high temperature is the same as that seen for the nutR⁺ phage.

That the failure of growth caused by the nutR62 mutation results from a deficiency in N modification was shown in another way. λnutR62 derivatives fail to grow in an Escherichia coli host with the nusA1 allele at 32°C. Previous studies (Tomich and Friedman,

Table 1. Effect of Rho and Temperature on Growth of λnutR⁻ r32

	K37 (Rho⁺)			K1024 (Rho⁻)	
	30°C	35°C	40°C	30°C	35°C
λcl857 r32	104	300	200	63	48
λnutR62 r32	90	1	<0.1	60	50

Data are expressed as the number of phages produced per infected cell. Burst sizes were taken from single-step growth curves, as described in the Experimental Procedures. The phages were not tested at 40°C in K1024 because K1024 is temperature-sensitive for growth.
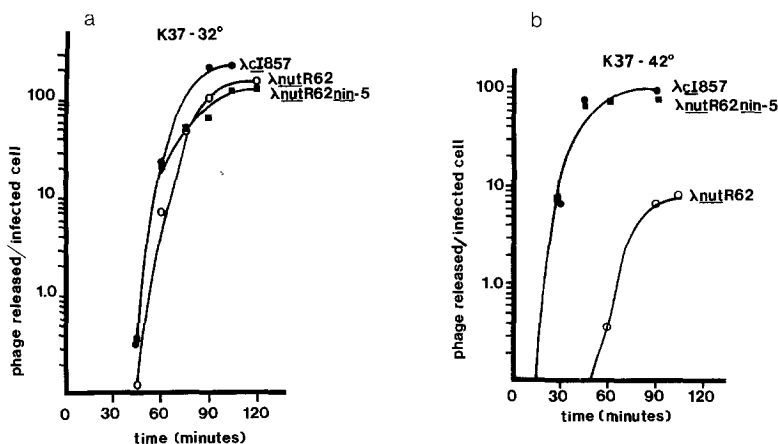
Figure 4. Effect of Temperature on the Growth of $\lambda nutR62$

Growth of λ (●——●), $\lambda nutR62$ (○——○) and $\lambda nutR62$ $nin5$ (■——■) was compared by single-step growth experiments in the Rho$^+$ host K37 at 32°C (a) and 42°C (b). Details of the growth experiments are given in the Experimental Procedures.

1977) have shown that lambda derivatives that for a variety of reasons require higher than normal levels of pN activity fail to grow in the $nusA1$ host at 32°C, even though they grow in nus^+ hosts under the identical conditions. This suggests that $\lambda nutR62$ fails to grow in the $nusA1$ host because the level of pN activity is too low in the $nusA1$ host to alter transcription effectively in the mutant Nut region.

Our finding that growth of both $\lambda nutR62$ and $\lambda nutR62$ $r32$ in Rho$^+$ hosts becomes progressively poorer at higher temperatures is consistent with previous studies showing that Rho-dependent termination is more active at higher temperatures (Das et al., 1976).

Isolation of Revertants of $\lambda nutR62$

Revertants of $nutR62$ were isolated, from a lysate of $\lambda nutR62$ $r32$, by selection for plaque-forming phages on a rho^+ host at 40°C. One revertant, $rev1$, was chosen for further study. Analysis by agarose gel electrophoresis of fragments generated by Hind III digestion showed that the revertant still has the IS2 insertion (data not shown).

Initial mapping placed the revertant mutation to the left of the $r32$–IS2 insertion. In this experiment, $\lambda nutR62$ $r32$ $rev1$ was crossed with $\lambda imm434$ $(QSR)_{80}$. Details of the mapping experiment are presented in Figure 5. One class of recombinant progeny commonly found, and therefore likely to result from a single recombination event, is $\lambda(QSR)_{80}$ that can grow in the $nusA1$ host at 32°C, a characteristic not found for phages carrying either $nutR62$ or the $r32$–IS2 insertion. Since such a recombinant is not found when the 434 phage is crossed with $\lambda nutR62$ itself, it is concluded that this class of recombinants contains the reversion. This mapping places the reversion to the left of the $r32$–IS2 insertion.

DNA Sequence Analysis of $nutR62$, $nutR37$, $nutR57$ and The Revertant

Cloned fragments containing the $nutR$ region from $nutR62$, $nutR37$, $nutR57$, its parent and the revertant, $rev1$, were prepared as outlined in the Experimental
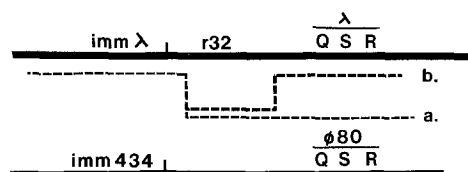

Figure 5. Mapping the $nutR$ Revertant with Respect to the $r32$

The $\lambda nutR62$ revertant was crossed with $\lambda imm434$ $(QSR)_{80}$, and recombinants that had crossed out the $r32$ but retained the revertant loci were selected with a $nusA$ $\lambda imm434$ lysogen at 32°C (k278); $\lambda r32$ derivatives fail to form plaques on $nusA1$ mutants under these conditions (Tomich and Friedman, 1977). Recombinants were then screened for the presence of $(QSR)_{80}$ by checking for growth on $himA$ at 32°C (A. Kikuchi and R. Weisberg, personal communication). The following strategy was used. (a) If the mutation is to the left of the $r32$, recombinants should be chiefly $(QSR)_{80}$. (b) If the mutation is to the right, most of the recombinants should be $(QSR)_\lambda$. When checked, 75% of the recombinants were $(QSR)_{80}$.

Procedures. Figure 6 shows restriction sites used in cloning the $nutR$ region. The DNA sequence of these cloned fragments was determined by the chain termination method of Sanger et al. (1977). Representative sequences are shown in Figure 7. In each case we determined the sequence from a site in cro to one in the $r32$–IS2. The parental phages showed no changes for this region from that reported for wild-type lambda by Rosenberg et al. (1978). Examination of the sequence obtained from the three $nutR$ mutants revealed in each case only one change, a deletion of one AT base pair in a run of what is normally seven AT base pairs that begin 15 bp upstream from the end of cro. Analysis of the revertant shows that it is a true reversion, since the only change from the sequence of $\lambda nutR62$ is the reestablishment of the run of seven AT base pairs.

From a functional point of view, the finding of three identical $nutR$ mutations in cro and the finding that the revertant is a true revertant were unexpected. From a structural point of view, however, the isolation of frameshift mutations in runs of AT base pairs was not unexpected. Previous work by Streisinger et al. (1966) showed that runs of AT base pairs can be hotspots for frameshift mutations.
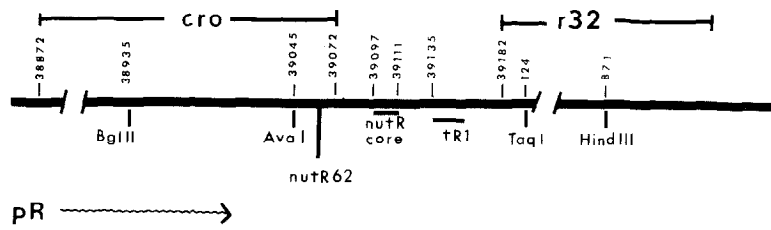
Figure 6. The *cro–nut–r32* Region

The region from P_R (pR) to the *r32*, showing the restriction sites used for cloning and identification of the genetic elements discussed in the text. Numbers (in nucleotides) correspond to the sequence determined by F. R. Blattner (personal communication). Wavy line: direction of transcription from P_R.
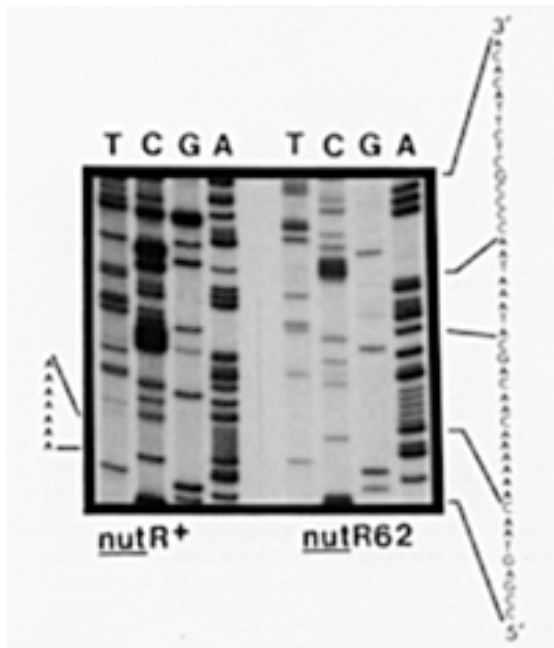

Figure 7. Sequencing Gel, Showing *nutR* Regions

(Left) The sequence from *nutR⁺*, extending from the Ava I site in *cro* to 46 bp beyond *cro*. (Right) The region from λ*nutR62*, extending from the Ava I site in *cro* to 19 bp beyond *cro*. The *nutR62* mutation is a deletion of one of the seven As shown on the left.

Discussion

We have established the facts that the selection we have developed yields mutations with the phenotype expected for a *nutR* mutation; pN fails to alter transcription initiating at P_R into a termination-resistant complex. As expected, genetic characterization of the mutations reveals that they map in the region between P_R and t_{R1} (see Figure 1), the region proposed to contain the site for pN modification of rightward transcription (Rosenberg et al., 1978). It was unexpected, however, that *nutR* mutations would be found in the 3′ end of the *cro* gene. Since the three *nutR* mutations analyzed by DNA sequencing all have a single-base-pair deletion in the same run of AT base pairs, we will focus our discussion on one of these deletions, *nutR62*, as the prototype of this class of mutations.

The *nutR62* deletion changes the reading frame of the *cro* gene so that an additional 3 bp are read prior to a translational stop codon. Isolation of a Nut⁺ revertant in which the proper reading frame is restored proves that the deletion in *nutR62* is responsible for the Nut⁻ phenotype. One way that the *nutR62* muta-

tion could reduce pN activity would be to alter a site directly involved in the action of pN or an associated host function. Since *nutR* and *nutL* function similarily, we expect that any sequence involved in pN action would be found in an analogous position in both of these Nut regions. Examination of the sequence of the analogous *nutL* region does not reveal any run of bases resembling the run of As where the *nutR62* deletion occurs. It therefore seems likely that this sequence is not directly involved in the pN reaction. We suggest two alternative ways that this change could result in the Nut⁻ phenotype: either the structural change in Cro itself is responsible, or the change in reading frame and the resultant change in the position of the terminating ribosome translating the *cro* gene influence the pN reaction.

We first consider the two likely ways that an altered Cro protein could cause a Nut⁻ phenotype. The altered Cro might either inhibit pN action or be unable to replace wild-type Cro in some part of the pN reaction. Our experiments with the induced lysogen carrying a λ*nutR62 r32* prophage argue against both of these possibilities. The fact that both λ and λ*cro⁻* grow in the induced λ*nutR62 r32* lysogen demonstrates that the altered Cro does not adversely influence pN action, and can function to replace the wild-type Cro. Thus the altered Cro is not likely the cause of the Nut⁻ phenotype of *nutR62*. Exclusion of these arguments leads us to the alternative explanation, that the Nut⁻ phenotype is due to some aspect of the progression of ribosomes past the normal translation termination sequence.

In considering how this misplacement of ribosomes might interfere with pN modification, we look at the sequences immediately downstream from *cro* (Figure 8). The "core" *nut* region of 17 bp of hyphenated dyad symmetry lies 24 bp from the 3′ terminus of *cro* (Rosenberg et al., 1978). In comparing sequences of the lambda *nut* regions (Rosenberg et al., 1978; Franklin and Bennett, 1979), we find that promoter proximal to the *nutL* (7 bp) and *nutR* (8 bp) core sequences is the sequence CGCTCTTA$_C^A$. Examination of the only other two *nut* region sequences available, *nutL* of P22 (A. Poteete, personal communication) and *nutR* of 21 (Schwarz, 1980) reveals similar sequences in the analogous positions. Downstream from 21 P_R, and 13 bp upstream from a region of hyphenated dyad symmetry that is similar, but not identical, to the λ*nut* core sequence, is the sequence TGCTCTTAA. Downstream from P22 P_L, and 10 bp upstream from

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                    Box 'A'                    CORE
 λnutL    ATGAAGGTGACGCTCTTAAAAATTAAGCCCTGAAGAAGGGCAGCATTCAAAGCAGAAGGCTT

 λnutR    TAAATAACCCCGCTCTTACACATTCCAGCCCTGAAAAAGGGCATCAAATTAAACCACACCTA

 21nutR   TAAGCAAATTGCTCTTTAACAGTTCTGGCCTTTCACCTCTAACCGGGTGAGCAAACATCAGC

 P22nutL  AACGCTCTTTAACTTCGATGATGCGCTGACAAAGCGCGAACAAATACCAAACGAGATTGGTT

 λqut     ATGGGTTAATTCGCTCGTTGTGGTAGTGAGATGAAAAGAGGCGGCGCTTACTACCGATTCCG
```
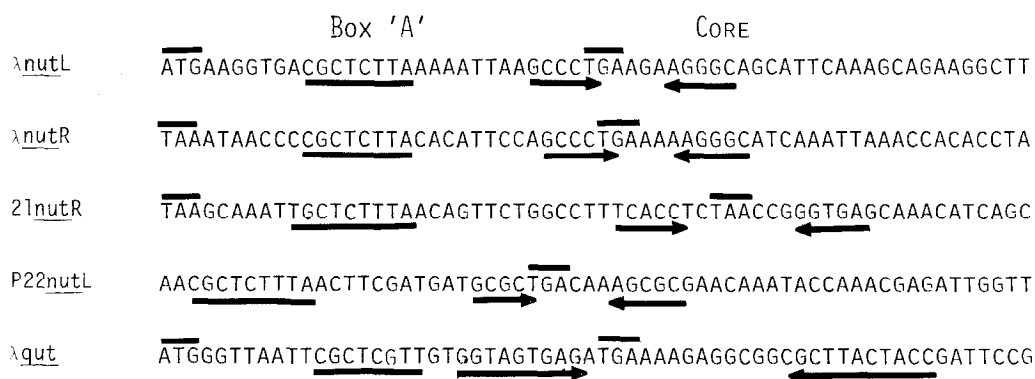
Figure 8. Comparison of Sequences in *nut* and *qut* Regions of Lambda and Related Phages

Compilation of *nut* regions and the putative region involved in the pQ reaction (*qut*). Converging arrows: regions of dyad symmetry thought to be sites that are specific for the action of pN (and perhaps pQ). Underlining: box A sequences. Lines above sequences: translation signals. The λ*nutR* sequence was from Rosenberg et al. (1978); the λ*nutL* sequence was from Franklin and Bennett (1979); the 21*nutR* sequence was from Schwarz (1980); the P22*nutL* sequence was from A. Poteete (personal communication); and the λ*qut* sequence was from Daniels and Blattner (1982).

the region of hyphenated symmetry again similar but not identical to the core λ*nut* sequence, is the sequence CGCTCTTTAA. Based on this data we have derived the consensus sequence CGCTCT(T)TAA, which will be referred to as box A. The fact that box A is held in common by all of the *nut* regions suggests that it serves a similar role in all of the pN reactions, perhaps recognizing a common factor. Since the various *nut* regions show specificity for their homologous N products (Friedman et al., 1973; Hilliker et al., 1978), such a common factor would most likely be something other than pN, perhaps one or more of the host Nus proteins (Friedman et al., 1981). The uniqueness of the core regions of each of the lambdoid phages is consistent with the hypothesis that these regions of hyphenated dyad symmetry play a role in the recognition of the various N functions, as previously proposed (Rosenberg et al., 1978).

Although there is no direct evidence for any host factor interaction with RNA or DNA at the box A sequence, we present circumstantial evidence that these sequences might be involved in NusA protein activity. The N products of phages lambda, 21 and P22, even though differing in the *nut* sites recognized, all require NusA protein for activity (Friedman et al., 1976; Hilliker et al., 1978). Moreover, another lambda antitermination protein, pQ, also requires NusA protein for its antitermination activity (Grayhack and Roberts, 1982). Transcription from $P_{R'}$, a promoter downstream from the Q gene, yields a 6S RNA in the absence of pQ. In the presence of pQ a large RNA is synthesized (Daniels and Blattner, 1982). Thus pQ permits transcription initiating at $P_{R'}$ to overcome termination signals (Forbes and Herskowitz, 1982). We have examined the sequence at the 5' end of the $P_{R'}$ 6S message (Daniels and Blattner, 1982), and have found a box A sequence. Fourteen base pairs from the 5' end of the 6S $P_{R'}$ RNA is the sequence CGCTCGTTGT. This sequence varies from the consensus box A sequence, having an additional G in the

center and no pair of As at the end. This box A sequence is 2 bp upstream from a region of hyphenated dyad symmetry that by analogy to pN–*nut*, could be the "*qut*," or pQ recognition, site.

Additional evidence supporting a role for the box A sequence in NusA activity can be found in studies on termination of transcription in bacterial operons. In vitro studies have shown that NusA protein is involved in transcription termination at the E. coli *trp t* terminator (Farnham et al., 1982), as well as in the E. coli *rrnB* leader region (Kingston and Chamberlain, 1981). Our examination of the sequences at the end of the *trp* operon (Wu and Platt, 1978; Wu et al., 1981) reveals the sequence CGCAGTTAA at the end of the last cistron of the *trp* operon, 33 bp from the *t* terminator. This sequence maintains the first 3 and the last 4 bp of the box A consensus sequence. The *rrnB* leader region contains the sequence TGCTCTTTAA (Brosius et al., 1981) 154 bp downstream from the P_I promoter and ~100 bp upstream from a NusA-dependent termination site (Kingston and Chamberlain, 1981). This sequence differs by 1 base (also a T at base 1) from the consensus box A sequence, and is identical to the box A sequence found in the *nutR* region of 21. Since this box A sequence is located in the conserved regions of all *rrn* genes that have been sequenced (de Boer et al., 1979; Young and Steitz, 1979), we suggest that the *rrnA, rrnD, rrnE* and *rrnX* operons will also show early termination that depends on NusA protein.

The identification of the box A sequence suggests one way that the frameshift created by *nutR62* might yield the Nut⁻ phenotype. Bound ribosomes protect from nuclease digestion approximately 8–10 bases downstream from the responsible triplet (Platt et al., 1976). In the case of *nutR*⁺, this would mean that the ribosome terminating *cro* translation would cover the bases up to, and possibly 1 base into, box A. However, in the case of the *nutR62* mutant, the ribosome terminating *cro* translation covers 4–5 bases into box A.

A ribosome stalling after termination could result in the unavailability of the box A site for interaction with a host function (NusA). Thus, even if the core sequence were available, full pN modification could not occur (Figure 9).

The data accumulated on pN action have not led to a definitive model of pN action. However, the finding that mutations in genes encoding ribosomal proteins influence the action of pN (Friedman et al., 1981; Ward and Gottesman, 1982) has led to the suggestion that the coupling of a ribosome to RNA polymerase could result in the formation of a termination-resistant transcription complex. If a ribosome terminating translation of *cro* can be expropriated for use in the pN reaction, then proper alignment of such a ribosome on the RNA might be essential for the coupling reaction. According to this scenario, the *nutR62* frameshift-induced misplacement of ribosomes would cause a misalignment that would abort the modification process. Consistent with such a role for ribosomes, we find translation signals at two positions in the various *nut* and *qut* regions (see Figure 8). First, 6–8 bp promoter-proximal to all but one of the box A sequences we find translation initiation or termination codons. Arguing against the significance of these upstream signals is the fact that no similar relationship of translation signals is found in P22*nutL*. Second,

translation termination signals can be found downstream from box A in each of the core regions of hyphenated dyad symmetry.

Although we are limited to speculation about the precise mechanism of the *nutR62* mutation, the fact that revertants regaining Nut activity restore the original *cro* sequence clearly identifies the *cro* mutation as that responsible for the Nut⁻ phenotype. Obviously the placement of *nutR* mutations in box A would be useful in proving its biological significance in the pN reaction. The run of As in *cro* where the *nutR* mutations are found is apparently a mutational hotspot, and we expect that most of the *nutR* mutations we isolate with our selection procedure will have changes in that run of As.

The effect of *nutR* mutations on lambda growth is temperature-dependent, exhibiting an increasingly greater inhibition on growth as the temperature increases. This suggests that more pN activity is required as the temperature increases. Considering the role of pN as a transcription-termination antagonist, these observations suggest that some aspect of the termination process might be more active at higher temperatures. Two observations are consistent with this interpretation. First, all of the host mutants that we have isolated that interfere with pN activity (*nus*) exhibit a temperature-sensitive phenotype, showing increased restriction on lambda growth as the temperature is increased. Unless we assume that every mutation in each of the *nus* genes results in a temperature-sensitive protein, these observations suggest a process in which more pN activity is required at higher temperature. Second, in vitro studies (Das et al., 1976; Richardson and Macy, 1981) show that the Rho-dependent termination and ATPase activity is more active at higher temperatures.
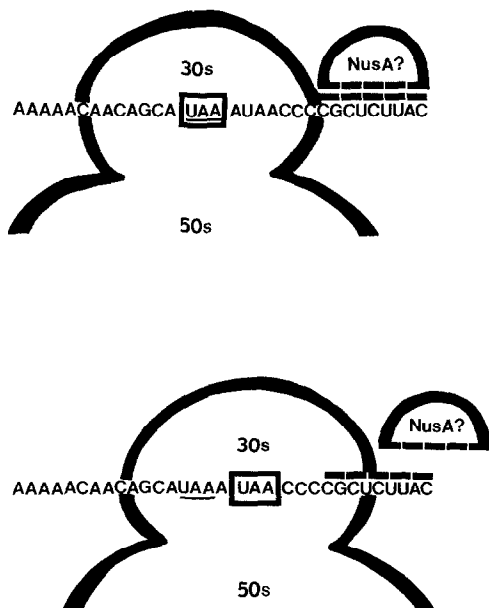

Figure 9. Model to Explain the Effect of the *nutR62* Mutation

(Top) The ribosome terminating at the normal *cro* UAA translation stop signal leaves box A free to recognize a factor or factors such as Nus A. If the ribosome is directly involved in the pN reaction, it could then be in the correct position, relative to other sites in the *nut* region, to participate in a reaction with pN or other factors, or both.

(Bottom) The ribosome terminating at a downstream UAA translation signal as a result of the *nutR62* frameshift mutation could sterically inhibit interactions of factors such as NusA with the RNA at box A. Alternatively, if the position of the terminating ribosome is important for the modification reaction, this misalignment could result in incomplete pN modification.

Experimental Procedures

Media and Growth of Phages
Media and methods for cell and phage growth have been described previously (Miller and Friedman, 1980). Phage crosses were carried out as described by Miller et al. (1980).

Strains
Strains used in this study are described in Table 2. In all experiments *nutR*⁻ phages also carried the *cI857* mutation. When this marker is irrelevant to the experiment, we have not listed it.

Complementation and Marker Rescue Assays
Two drops of a lawn of the lysogen to be tested were mixed with 2.5 ml of top agar and poured onto a tryptone broth plate. After the lawn had hardened, two or three different dilutions of each phage to be tested were spotted on the lawn. The plates were then incubated overnight at 42°C. If the prophage could complement the superinfecting phage, a zone of lysis could be observed where a dilute concentration of phage (10³/ml or less) was placed. If the prophage had the intact gene but could not express it, a zone of lysis occurred only where a more concentrated drop of phage (10⁶/ml or more) was placed.

Isolation of λ*nutR62* from λ*nutR62 r32*
λ*nutR62 r32* was crossed with λ*imm434* in K37 at 32°C. Recombinants that were λ*nutR62* were selected as normal plaque formers on

Table 2. Bacterial Strains and Phages

	Derived from	Relevant Genotype	Source
Bacterial Strains			
K37	W3102	*gal⁻* Str^R	M. Yarmolinsky
K95	K37	*nusA*	Our laboratory
K100	K37	*recA*	M. Gottesman
K159	K37	λ*imm434*	M. Yarmolinsky
K765	K95	*himA42 nusA1*	Our laboratory
K994	K100	*recA* (λ*cI857 r32 h80 Bam*)	This study
K1024		*rho ts15*	S. Adhya
1086	K1024	*rho ts15* (λ*imm434*)	Our laboratory
JM101		Used for M13 transformation	W. Dunnick
Phages			
λ*imm434 cI t1 N am7,53*		N⁻	M. Yarmolinsky
λ*imm434 cI2 O am29*		O⁻	R. Weisberg
λ*imm434 cI2 P am3*		P⁻	R. Weisberg
λ*cI857 cro27*		Cro⁻	M. Yarmolinsky
λ*imm21 P am3*		21P⁻	R. Weisberg
λ*imm434 cI2 cin1*		*cin1*	Our laboratory
λ*imm434 cI2*		*imm434*	M. Yarmolinsky
λ*cI857 r32 h80 Bam*		IS2 *cIII–int cI857*	Our laboratory

a λ*imm434* lysogen of K37 (K159) at 32°C. The Hind III restriction pattern confirmed that these recombinants do not carry the *r32*–IS2 element.

Single-Step Growth Curves

Cells were grown to a density of 10^8/ml in LB containing maltose and magnesium. Five milliliters of culture were pelleted and resuspended in 1 ml of 0.01 M MgSO₄. Phages were added at a multiplicity of infection of 0.1, incubated for 20 min at room temperature to allow adsorption and diluted so as to give a 1:10^4 dilution in a final volume of 20 ml of prewarmed LB. Aliquots of 1.0 ml were taken at specified times and incubated with 2 drops chloroform at 40°C for 20 min. Appropriate dilutions were then assayed for viable phages.

Cloning and Sequencing

All ligations were carried out at 16°C for 10–24 hr in 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM ATP and 1 U T4 DNA ligase. Bacteria were made competent for transformation as described by Cohen et al. (1972). Possible pBR322 or M13 clones were screened by isolating the plasmid or replicative form (Birnboim and Doly, 1979) and analyzing their restriction patterns on agarose or polyacrylamide gels. Enzymes were obtained from Bethesda Research Laboratories or New England BioLabs. Dideoxy sequencing was carried out according to the method of Sanger et al. (1977).

The *nutR* regions from λ*cI857 r32 Bam h80* (*nut⁺*), the λ*nutR62* revertant, λ*nutR37* and λ*nutR57* were cloned with phage DNA digested with Bgl II and Hind III and by ligation of the fragments into Bam HI–Hind III–digested M13mp9. JM101 was transfected, and clones containing an 871 bp piece from the Bgl II site in *cro* to the Hind III site in the *r32* were found and sequenced.

The *nutR* region from λ*nutR62* was cloned by digestion of the phage DNA with Ava I and Hind III and ligation of the fragments into Ava I–Hind III–digested pBR322. Amp^R Tet^S colonies were screened, and a clone containing the piece from the Ava I site in *cro* to the Hind

III site in *r32* was found. This plasmid was then digested with Taq I and Hpa II, and the pieces were ligated into the Acc I site of M13mp9. A clone containing the *nut* region was identified and sequenced.

References

Baumann, M. F. and Friedman, D. I. (1976). Cooperative effects of bacterial mutations affecting λ *N* gene expression. Virology *73*, 128–138.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmids. Nucl. Acids Res. *7*, 1513–1523.

Brachet, P., Eisen, H. and Rambach, A. (1970). Mutations of coliphage λ affecting the expression of replicative functions O and P. Mol. Gen. Genet. *108*, 266–276.

Brosius, J., Dulls, T. J., Sleeter, D. D. and Noller, H. (1981). Gene organization and primary structure of a ribosomal operon from *Escherichia coli*. J. Mol. Biol. *148*, 107–127.

Brunel, F. and Davison, J. (1975). Bacterial mutants able to partly suppress the effect of *N* mutations in bacteriophage λ. Mol. Gen. Genet. *136*, 167–180.

Calef, E. and Neubauer, Z. (1968). Active and inactive states of the cI gene in some λ defective phages. Cold Spring Harbor Symp. Quant. Biol. *33*, 765–767.

Cohen, S. N., Chang, A. C. Y. and Hsu, C. L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. Proc. Nat. Acad. Sci. USA *69*, 2110–2114.

Court, D. and Sato, K. (1969). Studies of novel transducing variants of lambda: dispensability of genes *N* and *Q*. Virology *39*, 348–352.

Couturier, M. and Dambly, C. (1970). Activation sequentielle des fonctions tardives chez les bacteriophages temperes. CR. Acad. Sci. Paris *270*, 428–430.

Dambly, C., Couturier, M. and Thomas, R. (1968). Control of development of temperate bacteriophages. II. Control of lysosyme synthesis. J. Mol. Biol. *32*, 67–81.

Daniels, D. L. and Blattner, F. R. (1982). The nucleotide sequence of the *Q* gene and the *Q* to *S* intergenic region of bacteriophage lambda. Virology *117*, 81–92.

Das, A., Court, D. and Adhya, S. (1976). Isolation and characterization of conditional lethal mutants of *Escherichia coli* defective in transcription termination factor rho. Proc. Nat. Acad. Sci. USA *73*, 1959–1963.

de Boer, H. A., Gilbert, S. F. and Nomura, M. (1979). DNA sequences of promoter regions for rRNA operons *rrn*E and *rrn*A in E. coli. Cell *17*, 201–209.

de Crombrugghe, B., Adhya, S., Gottesman, M. and Pastan, I. (1973). Effect of rho on transcription of bacterial operons. Nature New Biol. *241*, 260–264.

de Crombrugghe, B., Mudryj, M., DiLaura, R. and Gottesman, M. (1979). Specificity of the bacteriophage lambda *N* gene product (pN): *nut* sequences are necessary and sufficient for the antitermination by pN. Cell *18*, 1145–1151.

Eisen, H., Pereira da Silva, L. and Jacob, F. (1968). The regulation and mechanism of DNA synthesis in bacteriophage λ. Cold Spring Harbor Symp. Quant. Biol. *33*, 755–764.

Farnham, P. J., Greenblatt, J. and Platt, T. (1982). Effects of NusA protein on transcription termination in the tryptophan operon of Escherichia coli. Cell *29*, 945–951.

Forbes, D. and Herskowitz, I. (1982). Polarity suppression by the Q gene product of phage lambda. J. Mol. Biol., in press.

Franklin, N. C. and Bennett, G. N. (1979). The *N* protein of bacteriophage lambda, defined by its DNA sequence, is highly basic. Gene *8*, 107–119.

Friedman, D. (1971). A bacterial mutant affecting λ development. In The Bacteriophage Lambda, A. D. Hershey, ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 733–738.

Friedman, D. I. and Baron, L. S. (1974). Genetic characterization of a bacterial locus involved in the activity of the N function phage λ. Virology *58*, 141–148.

Friedman, D. I. and Gottesman, M. (1982). The lytic mode of λ development. In Lambda II, R. Hendrix, R. Weisberg, F. Stahl and J. Roberts, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), in press.

Friedman, D. I., Wilgus, G. S. and Mural, R. J. (1973). Gene N regulator function of phage λ*imm*21: evidence that a site of N action differs from a site of N recognition. J. Mol. Biol. *81*, 505–516.

Friedman, D. I., Baumann, M. and Baron, L. S. (1976). Cooperative effects of bacterial mutations affecting λ *N* gene expression. I. Isolation and characterization of a *nus*B mutant. Virology *73*, 119–127.

Friedman, D. I., Schauer, A. T., Baumann, M. R., Baron, L. S. and Adhya, S. L. (1981). Evidence that ribosomal protein S10 participates in the control of transcription termination. Proc. Nat. Acad. Sci. USA *78*, 1115–1119.

Georgopoulos, C. P. (1971a). A bacterial mutation affecting N function. In The Bacteriophage Lambda, A. Hershey, ed. (Cold Spring Harbor), New York: Cold Spring Harbor Laboratory), pp. 639–645.

Georgopoulos, C. P. (1971b). Bacterial mutants in which the gene N function of bacteriophage lambda is blocked have an altered RNA polymerase. Proc. Nat. Acad. Sci. USA *68*, 2977–2981.

Ghysen, A. and Pironio, M. (1972). Relationship between the *N* function of bacteriophage λ and host RNA polymerase. J. Mol. Biol. *65*, 259–272.

Gottesman, M. and Yarmolinsky, M. (1968). Integration-negative mutants of bacteriophage lambda. J. Mol. Biol. *31*, 487–505.

Grayhack, E. J. and Roberts, J. W. (1982). The phage λ Q gene product: activity of a transcription antiterminator in vitro. Cell *30*, 637–648.

Greenblatt, J., Malnoe, P. and Li, J. (1980). Purification of the gene N transcription anti-termination protein of bacteriophage λ. J. Biol. Chem. *255*, 1465–1470.

Greer, H. (1975). The kill gene of bacteriophage lambda. Virology *66*, 589–604.

Herskowitz, I. and Signer, E. R. (1970). Control of transcription from the *r* strand of bacteriophage lambda. Cold Spring Harbor Symp. Quant. Biol. *35*, 355–368.

Hilliker, S. and Botstein, D. (1976). Specificity of genetic elements controlling regulation of early functions in temperate bacteriophages. J. Mol. Biol. *106*, 537–566.

Hilliker, S., Gottesman, M. and Adhya, S. (1978). The activity of Salmonella phage P22 gene 24 product in *Escherichia coli*. Virology *86*, 37–47.

Inoko, H. and Imai, M. (1976). Isolation and genetic characterization of the *nit*A mutants of *E. coli* affecting the termination factor Rho. Mol. Gen. Genet. *143*, 211–221.

Kaiser, A. D. and Jacob, F. (1957). Recombination between related temperate bacteriophages and the genetic control of immunity and prophage localization. Virology *4*, 509–521.

Keppel, F., Georgopoulos, C. and Eisen, H. (1974). Host interference with expression of the lambda N gene product. Biochimie *56*, 1503–1509.

Kingston, R. E. and Chamberlain, M. J. (1981). Pausing and attenuation of in vitro transcription in the *rrn*B operon of E. coli. Cell *27*, 523–531.

Kourilsky, P., Perricaudet, M., Gros, D., Garapin, A., Gottesman, M., Fritsch, A. and Tiollais, P. (1978). Description and properties of bacteriophage lambda vectors useful for the cloning of EcoRI DNA fragments. Biochimie *60*, 183–187.

Miller, H. I. and Friedman, D. I. (1980). An Escherichia coli gene product required for lambda site specific recombination. Cell *20*, 711–719.

Miller, H. I., Mozola, M. A. and Friedman, D. I. (1980). *int*-h: an *int* mutation of phage λ that enhances site-specific recombination. Cell *20*, 721–729.

Platt, T., Squires, C. and Yanofsky, C. (1976). Ribosome-protected regions in the leader–trpE sequence of *Escherichia coli* tryptophan messenger RNA. J. Mol. Biol. *103*, 411–420.

Richardson, J. P. and Macy, M. R. (1981). Ribonucleic acid synthesis termination protein ρ function: effects of conditions that destabilize ribonucleic acid secondary structure. Biochemistry *20*, 1133–1139.

Richardson, J. P., Fink, P., Blanchard, K. and Macy, M. (1977). Bacteria with defective Rho factors suppress the effects of N mutations in bacteriophage λ. Mol. Gen. Genet. *153*, 81–85.

Roberts, J. W. (1969). Termination factor for RNA synthesis. Nature *224*, 1168–1174.

Rosenberg, M. and Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. Ann. Rev. Genet. *13*, 319–353.

Rosenberg, M., Court, D., Shimatake, H., Brady, C. and Wulff, D. L. (1978). The relationship between function and DNA sequence in an intercistronic regulatory region of phage λ. Nature *272*, 414–423.

Salstrom, J. S. and Szybalski, W. (1978). Coliphage *nut*L⁻: a unique class of mutants defective in the site of gene N product utilization for antitermination of leftward transcription. J. Mol. Biol. *124*, 195–221.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. USA *74*, 5463–5467.

Schwarz, E. (1980). Sequenz analyse des DNA lambdoide bacteriophages. Ph.D. thesis, University of Freiburg, Freiburg, Federal Republic of Germany.

Sternberg, N. (1976). A class of rif^R polymerase mutations that interfere with the activity of coliphage N gene product. Virology *73*, 139–154.

Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E. and Inouye, M. (1966). Frameshift mutations and the genetic code. Cold Spring Harbor Symp. Quant. Biol. *31*, 77–84.

Sussman, R. and Jacob, F. (1962). Sur un systeme de repression thermosensible chez le bacteriophage λ d'*Escherichia coli*. Compt. Rend. Acad. Sci. *254*, 1517–1519.

Thomas, R. (1966). Control of development in temperate bacteriophages. I. Induction of prophage genes following hetero-immune super-infection. J. Mol. Biol. *22*, 79–95.

Thomas, R., Leurs, C., Dambly, C., Parmentier, D., Lambert, L., Brachet, P., Lefebvre, N., Mousset, S., Porcheret, J., Szpirer, J. and Wauters, D. (1967). Isolation and characterization of new *sus* (amber) mutants of bacteriophage λ. Mutation Res. *4*, 735–741.

Tomich, P. K. and Friedman, D. I. (1977). Isolation of mutations in insertion sequences that relieve IS induced polarity. In DNA Insertion Elements, Plasmids and Episomes, S. Adhya, A. I. Bukhari and J. A. Shapiro, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 99–107.

Ward, D. F. and Gottesman, M. E. (1982). Suppression of transcription termination by phage lambda. Science *216*, 946–951.

Williams, J. G. K., Wulff, D. L. and Nash, H. A. (1977). A mutant of *Escherichia coli* deficient in a host function required for phage lambda integration and excision. In DNA Insertion Elements, and Episomes,

S. Adhya, A. I. Bukhari and J. A. Shapiro, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 357–361.

Wu, A. M. and Platt, T. (1978). Transcription termination: Nucleotide sequence at 3' end of tryptophan operon in *Escherichia coli*. Proc. Nat. Acad. Sci. USA *75*, 5442–5446.

Wu, A. M., Christie, G. E. and Platt, T. (1981). Tandem termination sites in the tryptophan operon of *Escherichia coli*. Proc. Nat. Acad. Sci. USA *78*, 2913–2917.

Wulff, D. L. (1976). Lambda *cin*-1, a new mutation which enhances lysogenization by bacteriophage lambda, and the genetic structure of the lambda *cy* region. Genetics *82*, 401–416.

Young, R. A. and Steitz, J. A. (1979). Tandem promoters direct E. coli ribosomal RNA synthesis. Cell *17*, 225–234.