J. Mol. Biol. (1987) 194, 679–690

λ N Antitermination System: Functional Analysis of Phage Interactions with the Host NusA Protein

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(Received 17 March 1986, and in revised form 22 October 1986)

Coliphage λ gene expression is regulated temporally by systems of termination and antitermination of transcription. The λ-encoded N protein (pN) acting with host factors (Nus) at sites (*nut*) located downstream from early promoters is the first of these systems to operate during phage development. We report observations on some of the components of this complex system that, in part, address the way in which these elements interact to render RNA polymerase termination-resistant. (1) The isolation of a conditionally lethal cold-sensitive *nus*A mutation demonstrates that NusA is essential for bacterial growth. (2) The effect on λ growth in a host in which the *Salmonella* NusA protein is overproduced suggests that NusA is essential for *N*-mediated antitermination in phage λ. (3) A truncated NusA product, representing only the amino two-thirds of the native protein, is active for both bacterial growth and pN action, indicating that the carboxy end of the molecule may not be a functionally important region. (4) λ pN can function with the heterologous *nut* region from *Salmonella typhimurium* phage P22 when λ pN is overproduced, demonstrating that λ pN can function with the *nut* regions of other lambdoid phages. (5) A single base-pair change in the λ *nut*R *box*A sequence that was selected to permit a λ derivative to utilize the *Salmonella* NusA protein restores λ growth in the *Escherichia coli* *nus*A1 host.

1. Introduction

Temporal regulation of coliphage λ gene expression is imposed primarily by a system of transcription termination and antitermination (for a review, see Friedman & Gottesman, 1983). Antitermination of the early λ transcripts (Roberts, 1969) results from the action of a small, highly basic phage protein, pN (Franklin & Bennett, 1979; Greenblatt & Li, 1982). Details of pN antitermination are shown diagrammatically in Figure 1 and are reviewed by Friedman & Gottesman (1983). In brief, transcription initiating at the early λ promoters, *p*L and *p*R, becomes termination-resistant when pN and bacterial factors (Nus proteins) act at sites (*nut*) to alter RNA polymerase activity. Subsequent transcription downstream from *nut* becomes resistant to many, but not all, transcription termination signals. λ derivatives that are *N*-independent have been isolated; one class of these mutants (*λnin*) is deleted for the *t*R2 and *t*R3 termination regions (Court & Sato, 1969; Fiandt *et al.*, 1971; K. Leason & D. I. Friedman, unpublished results; M. Gottesman, personal communication). The products of five *Escherichia coli* genes have been identified as Nus factors (Friedman & Gottesman, 1983).

The *nus*A gene maps at minute 69 on the *E. coli* chromosome and was identified in studies employing an *E. coli* K12 host with the *nus*A1 mutation (Friedman, 1971; Friedman & Baron, 1974). This mutation fails to support λ growth at 42 °C, because it results in a severe reduction in pN action. At lower temperatures wild-type λ grows well, even though there is reduced pN activity (Friedman *et al.*, 1973*a*). The *nus*A gene encodes a 54,000 M_r protein (determined from sequence; Ishii *et al.*, 1984; Y. Nakamura, personal communication) that binds both to RNA polymerase and to pN

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0022-2836/87/080679-12 $03.00/0

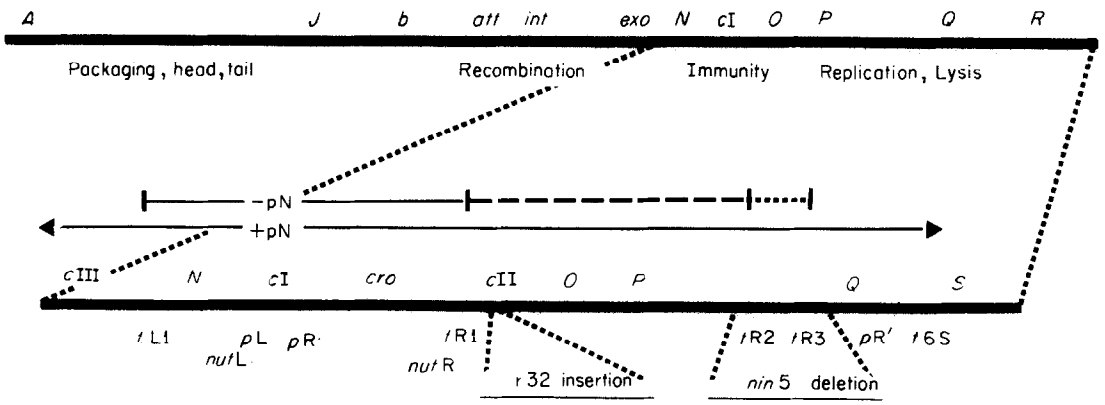

Figure 1. Lambda and transcription antitermination. The linear phage chromosome depicted in the upper part of the Figure is drawn approximately to scale (Szybalski & Szybalski, 1979). Only some genes are shown. The bottom diagram is a genetic map of the bacteriophage λ immunity and replication regions (not to scale). The repressor gene, *c*I, lies between the 2 early promoters, *p*L and *p*R, and its protein product represses this transcription during lysogeny by binding to the operators *o*L and *o*R. During execution of the lytic program, transcripts terminate at *t*L1 on the left, partially at *t*R1 on the right, and more completely at *t*R2 and *t*R3. Once N protein has achieved a sufficient concentration, it acts at the *nut*L and *nut*R regions. Action at *nut*R results in transcription that proceeds through the terminators and into *Q*, the gene encoding the late transcription antiterminator. pQ action permits transcripts to reach the distal morphogenic genes. Also shown is the r32-IS*2* insertion in the λ *c*II gene as well as the *nin*5 deletion that relieves λ of *N*-dependency by removal of the *t*R2 and *t*R3 terminators. The primary transcripts that are made in the presence or absence of pN are depicted by the lines just above the bottom diagram.

(Greenblatt & Li, 1981), although typically it runs at an apparent molecular weight of 69,000 on polyacrylamide gels. In a variety of transcriptional systems. NusA enhances the pausing of RNA polymerase as well as transcription termination and antitermination (Greenblatt *et al.*, 1981; Kingston & Chamberlin, 1981; Farnham *et al.*, 1982; Lau *et al.*, 1982; Simons & Kleckner, 1983; Fisher & Yanofsky, 1983; Schmidt & Chamberlin, 1984; Peacock *et al.*, 1985; Sharrock *et al.*, 1985).

The construction of a viable *E. coli–Salmonella typhimurium* hybrid host showed that the *Salmonella nus*A gene, when expressed in *E. coli*, can supply any NusA activity that is essential for *E. coli* growth (Baron *et al.*, 1970; Friedman & Baron, 1974). This hybrid bacterium carries a small *Salmonella* DNA substitution in the *E. coli* chromosome; it expresses the wild-type *Salmonella nus*A gene (*nus*A^{Sal}) instead of the *E. coli* gene. The NusA^{Sal} protein does not support growth of wild-type λ. As in the case of the *nus*A1 mutant, the hybrid bacterium (hereafter called the NusA^{Sal} host) does not support the growth of *N*-dependent λ derivatives, but supports the growth of *N*-independent phages such as λ*nin*.

Nakamura *et al.* (1986) have isolated mutations in *nus*A that result in the death of the bacterium at high temperature. These results suggest that *nus*A is an essential function for growth at high temperature. A number of experiments demonstrate a difference in the requirement for Nus factors at high and low temperature in the pN-mediated antitermination reaction (Friedman & Gottesman, 1983), raising the question of whether NusA protein is also required for bacterial growth at low temperature.

The λ *nut* regions, where pN and Nus factors alter RNA polymerase activity, are located downstream from the *p*L and *p*R promoters, but upstream from the terminators (Fig. 1; Friedman *et al.*, 1973*b*; Adhya *et al.*, 1974; Franklin, 1974; Salstrom & Szybalski, 1978). Mutational analyses have demonstrated functional roles for two sequences in the *nut* region: the *nut* stem–loop structure (a 17-base-pair sequence also referred to as *box*B), and a seven base-pair sequence called *box*A. The first *nut* mutations were identified in the *box*B region (Salstron & Szybalski, 1978). Subsequently, two types of mutation in *box*A were identified: (1) the *box*A1 allele that enables a λ derivative to use NusA^{Sal} (Friedman & Olson, 1983); and (2) two other changes in *box*A that reduced pN-mediated antitermination, demonstrating that an intact *box*A sequence is required (at least at 40 to 42°C) for the pN reaction (Olson *et al.*, 1984; Peltz *et al.*, 1985). Identification of the A to T transversion in *box*A1 led to the suggestion that *box*A could be a NusA target site (Friedman & Olson, 1983).

The *box*A1 mutation was obtained by selecting for a λ mutant that could grow in the NusA^{Sal} host. A two-stage selection procedure was devised, based upon the results of studies *in vivo* and *in vitro* on pN interaction with NusA. A λ derivative was employed that has an *N* mutation (*pun*A1) that permits phage growth in an *E. coli nus*A1 mutant at 42°C. It was assumed that *pun*A1 results in the expression of a more active pN. The phage also has an IS*2* element (the r32 insertion) located immediately downstream from *nut*R (Fig. 1; Brachet *et al.*, 1970; Tomich & Friedman, 1977). The strong ρ-dependent termination signal contributed by r32-IS*2* (de Crombrugghe *et al.*,

1973) was necessary to eliminate other classes of mutations that could make λ growth *N*-independent (discussed by Salstrom & Szybalski, 1978). The first step in the mutant selection yielded a phage that made a tiny plaque on the NusA^{Sal} host at 37 °C. This phage carries a second mutation in *N*: *pun*A133 (originally called *pun*A*).

The λ*pun*A1,133 phage was used in the second round of selection to generate a mutant that forms normal-sized plaques at 42 °C on the *nus*A^{Sal} host; the mutation responsible for this phenotype was mapped to the *nut*R region. DNA sequencing identified the responsible change in the *box*A region: a single base-pair transversion resulted in the *box*A sequence 5′-CGCTCTTT compared to the wild-type CGCTCTTA. This change had been predicted on the basis of the knowledge that other lambdoid pN-like proteins that are active with NusA^{Sal} (expressed by phages P22 and 21) have the three T residues at the 3′ end of their *box*A sequences (Friedman & Olson, 1983).

This paper reports the results of a genetic dissection of λ*pun*A1,133 *box*A1; the *N* (*pun*A) mutations and *nut*R (*box*A) mutation were separated from each other and studied independently. We have found that the mutant pN protein and the altered *box*A sequence each contribute an enhancement in phage growth, not only with the *Salmonella* NusA protein, but also with the *E. coli nus*A1 gene product. During the course of this analysis, we also: (1) obtained a conditionally lethal *E. coli nus*A mutation; (2) cloned and overproduced λ pN protein; and (3) cloned and overproduced *Salmonella* NusA protein. The results of these experiments prove that NusA protein is essential for antitermination and are consistent with the idea that *box*A might be a NusA recognition signal.

2. Materials and Methods

(a) *Bacteria, phage and plasmids*

Bacteria, phage and plasmid constructs are listed in Table 1.

(b) *Genetic techniques*

Media and basic genetic methods were as described (Miller & Friedman, 1980; Miller *et al.*, 1980). Mutagenesis with a *mut*D5 *E. coli* strain was carried out as described by Friedman & Olson (1983).

(c) *Nucleic acid techniques*

DNA preparation, restriction digests, ligations and transformations were performed essentially according to Maniatis *et al.* (1982).

(d) *Isolation of* nus*Acs10*

The directed mutagenesis method of Hong & Ames (1971) was used. Phage P1 was grown on an *E. coli* host, K1456 (a *nus*A$^+$ strain), that carries a Tn*5* element in *arg*G. The *arg*G gene is >95% contransductable with *nus*A (Friedman & Baron, 1974). The resulting P1 lysate was concentrated and treated with hydroxylamine, as described by Hong & Ames (1971). The treated P1 lysate was used to infect *E. coli* strain K37, and kanamycin-resistant (Kan^r) transductants were isolated. Two of the transductants obtained grew at 40 °C, but failed to grow at temperatures below 30 °C. One, K1914, was chosen for further study.

(e) *Construction of* N *clones*

DNA fragments containing the *N* genes of λ*c*I857 and λ*c*I$^-$*pun*A1,133 r32 were purified from agarose as

Table 1
Bacteria, phage and plasmids

Strain	Relevant genotype	Source
A. E. coli *K12*		
K37	W3102 *nus*$^+$ *str*^{R} (*nus*$^+$ parent)	NIH collection
K95	*nus*A1	This laboratory
K450	*nus*B5	This laboratory
K554	*nus*C60	This laboratory
K556	*nus*E71	This laboratory
K1102	*nus*A^{Sal}	This laboratory
K1456	*arg*G::Tn*5*	This work
K1479	*nus*A1 *nus*E71	This laboratory
K1863	JM101	J. Messing/ W. Dunnick
K1914	*nus*A*cs*10	This laboratory
K2065	K1914 (λ*imm*21*nus*A$^+$)	This work
K2067	K1914 (λ*imm*21*nus*A1)	This work
K2166	(λ[BAM]$_\Delta$*N*$^+$cI14[H1]$_\Delta$)	S. Adhya/D. Ward
K2289	JM83/pUC9	BRL, Inc
K2415	*rec*A1 *nus*A*cs*10	This work
K2417	K2415/pWR324	This work
K2423	K1863/pNAS100	This work
K2429	K1863/pNAS110	This work
K2448	*rec*A1 *nus*A1	This work
K2449	K2448/pWR324	This work
K2662	K37/pDPT429	D. Taylor
K2664	K37/pNAS200	This work
K2668	K37/pNAS150	This work
K2719	K1863/mp9-*Npun*A1,133	This work
K2898	K1914/pNAS1000	This work
K3223	K1102/pNAS1000	This work

Genotype	Source
B. *Phage*	
λ*c*I857	NIH collection
λamA11	NIH collection
λamR5	NIH collection
λamQ73,501	NIH collection
λ*bio*256*sex*	NIH collection
λ*bio*256*sex nin*5	This laboratory
λ*pun*A1	This laboratory
λ*c*I60	NIH collection
λ*nin*5	NIH collection
λ1059*arg*G^{Sal} *nus*A^{Sal}	This work
λr32	This laboratory; made from λ*N*am7,53 r32 from W. Szybalski
λr32 *pun*A1	This laboratory
λr32 *pun*A1,133	This laboratory
λr32 *pun*A1,133 *box*A1	This laboratory
λ*box*A1	This work
λr32 *box*A1	This work
λ*imm*21 *nus*A$^+$ *nin*5	S. Adhya
λ*imm*21 *nus*A1 *nin*5	S. Adhya
λ*imm*21 *nus*A$^+$	This laboratory; made from λ*imm*21 *nus*A$^+$ *nin*5
λ*imm*21 *nus*A1	This laboratory; made from λ*imm*21 *nus*A1 *nin*5
λ*imm*P22 12am*N*11	S. Hilliker & D. Botstein
λ*imm*P22 24am*S*4	S. Hilliker & D. Botstein

*Bam*HI–*Pvu*I restriction fragments of λ DNA. These fragments were then cleaved with *Hae*III and cloned into the *Sma*I site of M13mp9 (Messing, 1983) and sequenced using the chain termination method as described by Biggin *et al.* (1983). Because there are *Hae*III sites adjacent to both the 5′ and 3′ ends of the *N* gene, this cloning yielded a fragment with only a few bases on each side of the *N* coding region, thus eliminating the *nut*L site. The presence of *nut*L on the plasmid could conceivably interfere with the cloning by causing plasmid transcripts to antiterminate, thereby leading to overproduction of a product that is toxic in large amounts. The use of *Hae*III also prevents translational read-through into *N* from upstream by introducing a stop codon in the *lacZ* frame. Clones which contained the *N* gene in the correct orientation with respect to the *lac* promoter were identified by DNA sequencing.

The N^+ gene was transferred to both high and low copy expression vectors. Cloning into the pBR322 plasmid derivative pUC9 (Vieira & Messing, 1982) was accomplished by cutting the M13mp9 clones with *Eco*RI and *Hin*dIII enzymes and transferring them into the same sites in pUC9. pUC9 is identical with M13mp9 throughout the *lac* and polylinker regions. The copy number of pUC9 was observed to be far above the expected values for a pBR322 derivative (data not shown); this is because the *rop* gene, which is involved in the control of DNA synthesis (Caesareni *et al.*, 1982), was partially deleted during vector construction (Vieira & Messing, 1982). pNAS150 is the multicopy plasmid encoding pN^+.

The entire *p*lac-*N* constructions were transferred from the M13 clones to a single copy plasmid, pDPT429 (an R-factor derivative; D. Taylor, personal communication). This was accomplished by cutting the M13mp9 clones with *Ava*I and *Eco*RI endonucleases, ligating with *Eco*RI-cut pDPT429, filling out the unpaired *Eco*RI and *Ava*I sites, and ligating again. The N^+ clone was named pNAS200.

(f) *Isolation of the* Salmonella nus*A gene*

Earlier genetic studies demonstrated that the *Salmonella arg*G gene can be used as a selective marker to transfer *nus*A^{Sal} into an *E. coli arg*G^- auxotroph (Baron *et al.*, 1970; Friedman & Baron, 1974). *arg*G^+ transducing phage were selected from a library (constructed by R. Maurer) of *Salmonella* DNA cloned into λ1059 (Karn *et al.*, 1980). The selection was done by mixing dilutions of the phage bank with a culture of *E. coli* K12 *arg*G::Tn*5* in 10 mM-$MgSO_4$ and then plating onto M9 glucose minimal medium along with 0·1 ml of LB (Luria–Bertani medium) broth in 2·5 ml agarose. After 2 days, large-haloed plaques could be seen on the slightly grown bacterial lawn. These plaques appeared at the expected frequency of 2×10^{-3}. The plaques were purified under the selection conditions and were then used to make standard λ lysates.

Phage carrying the *Salmonella nus*A gene were identified in the collection of *arg*G clones by screening for complementation of the *E. coli nus*A*cs*10 allele for growth at low temperature. Bacteria carrying the *nus*A*cs*10 mutation are cold-sensitive for cell growth. The λ1059 clones are deleted for functions essential for lysogeny, so lysogens were obtained by homologous recombination into a resident λ prophage. Colonies were selected as *arg*G^+ prototrophs and were purified on minimal medium at 40 °C. They were then shifted to 30 °C to test for complementation of the cold-sensitive phenotype. Two of 8 *arg*$^+$ lysogens restored wild-type growth characteristics to the *nus*A*cs*10 bacterium at 30 °C.

(g) *Construction of pNAS1000*

One of the λ1059 *arg*G^{Sal} *nus*A^{Sal} clones was chosen for subcloning. The phage DNA was partially digested with Sau3A under conditions that yielded overlapping fragments of an optimal size range. Fragments ranging in size from $1{\cdot}5 \times 10^3$ to $4{\cdot}0 \times 10^3$ base-pairs were eluted from an agarose gel and were ligated into the *Bam*HI site of pACYC184. Because the transducing phage studies showed that *nus*A^{Sal} could complement a host that expresses the gene for the cold-sensitive NusA10 protein, the ligated DNA was transformed into such a bacterium (K1914). The cells were grown at the permissive temperature and then were shifted to low temperature. After several hours there was good growth in the experimental culture, but not in a control culture that was transformed with recircularized vector. Samples of the culture were plated onto chloramphenicol plates (to select for transformants) at 30 °C (to demand complementation of *nus*A*cs*10). Resulting colonies were purified; one that was chosen for further analysis carries an insert of $2{\cdot}8 \times 10^3$ base-pairs (pNAS1000).

(h) *DNA sequencing*

The chain termination method of Biggin *et al.* (1983) was employed with the following modifications. JM101 was grown overnight in minimal (M9) glucose medium. Two-ml tubes of LB were inoculated with 1 drop of the overnight culture, plus a single purified plaque. These were incubated at 37 °C with shaking for 6 h, and then 1·5 ml of cells were pelleted in microfuge tubes for 10 min. One ml of supernatant was transferred to tubes containing 200 μl of 20% (w/v) polyethylene glycol, 2·5 M-NaCl, and incubated at room temperature for 15 min. After a 10-min microfuge spin, the supernatant was completely removed from the pellet. The pellet was resuspended in 150 μl of TE buffer (10 mM-Tris·HCl (pH 8·0), 1 mM-EDTA), 50 μl of phenol were added and tubes were vortexed and microfuged for 15 min. Then 100 μl of the aqueous phase was precipitated in the presence of 300 μl of ethanol and 10 μl of 3 M-NaOAc (pH 5·5), at 70 °C. The nucleic acid was pelleted by a 15-min spin, rinsed twice with 70% ethanol (2 × 4 min spins), dried, and resuspended in 40 μl of TE buffer.

Following incubation of the sequencing reactions, samples were heated to 100 °C for 5 min, quenched on ice, and loaded onto 6% (w/v) polyacrylamide gels containing 8 M-urea, 50 mM-Tris-borate (pH 8·1), 1 mM-EDTA. One glass plate was treated with γ-methacryloxypropyl-trimethoxysilane (Mallinckrodt) to permit the gel to be dried onto the plate. Gels were fixed (10% (v/v) acetic acid, 10% (v/v) methanol for 20 min), rinsed (water for 2 × 10 min) and dried (80 °C for 50 min) and then autoradiographed.

(i) *Construction of* λbox*A1*

We replaced the mutant *N* gene in λ*pun*A1,133 *box*A1 with a wild-type *N*. A *rec*$^+$ host, K37, that carries pNAS150 (N^+) was infected with λ*pun*A1,133 *box*A1. The resulting lysate was plated on a mixed bacterial lawn composed of *E. coli* strains K37 (*nus*A^+) and K1102 (*nus*A^{Sal}) in a ratio of 1 : 5. We expected that λ*box*A1 would not grow on a lawn of K1102 at 40 °C because earlier experiments showed that it was not possible to

isolate single-step mutations mapping in the right operon of λr32 *pun*A1 that would permit growth in K1102. We could screen for loss of growth on K1102 by using a mixture of bacteria, because λ*pun*A1,133 *box*A1 would grow on both hosts, thus making clear plaques at 40°C. Phage that do not grow on K1102 (the expected phenotype of λN^+*box*A1) plate turbid (due to unlysed K1102 in a K37 zone of lysis). We found turbid plaques at a frequency of $\sim 10^{-2}$, compared with $< 10^{-4}$ for a control using the parent vector pUC9 alone. To prove that *box*A1 was the only phage mutation responsible for the phenotype of λ*box*A1, a phage was constructed by crossing wild-type λ with a fully sequenced M13 clone carrying, as the only change from wild-type, the *box*A1 mutation.

(j) *Immuno-electroblotting*

Protein extracts were prepared as described by Ames & Nikaido (1976) and subjected to electrophoresis in SDS/polyacrylamide gel electrophoresis (7·5% gel). Transfer to nitrocellulose paper (NCP) was done essentially as described by Towbin *et al.* (1979). Blots were blocked with a solution of 3% (w/v) bovine serum albumin (BSA) in TBS (0·5 M-NaCl, 20 mM-Tris·HCl (pH 7·5)). The NCP was incubated with agitation overnight in rabbit anti-NusA serum (1 : 500 dilution in 1% BSA/TBS) at room temperature. After 1 wash in water and 2 washes in TBS, the blot was then exposed to *Staphylococcus aureus* protein A conjugated with horseradish peroxidase (1 : 2000 dilution in a 1% BSA/TBS solution). The NCP was washed as described above and immune complexes were detected by treatment with H_2O_2 and 4-chloro-1-naphthol.

For preparation of immune serum, rabbits were immunized intradermally with purified *E. coli* NusA protein emulsified in complete Freund's adjuvant and boosted by intradermal injection of NusA protein in incomplete Freund's adjuvant. Serum from all rabbits reacted positively with purified NusA protein in an enzyme-linked immunoadsorbent assay. Molecular weights were determined using Bethesda Research Laboratories' prestained protein molecular weight standards (myosin, 200,000; phosphorylase B, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700).

3. Results

(a) *Isolation of a conditionally lethal* nus*A mutation*

To extend the characterization of the *nus*A gene product's role in bacterial growth as well as to facilitate the cloning of the *nus*A^{Sal} gene, we looked for conditionally lethal *nus*A mutations. We exploited the fact that *nus*A is located near *arg*G (minute 69 on the *E. coli* chromosome). A Tn*5* transposon, which confers resistance to the antibiotic kanamycin (Kan^r), was placed in the *arg*G gene to provide an easily selectable genetic marker. A lysate of phage P1 grown on this host and mutagenized *in vitro* with hydroxylamine was used to transduce a nus^+ host to Kan^r. These transductants were screened for conditional lethality, i.e. failure to grow at 30°C. We report on one mutation isolated in this manner; to simplify the discussion, we anticipated the results of the mapping studies described below and will refer to the mutation as *nus*A*cs*10.

E. coli carrying the *nus*A*cs*10 mutation grow well above 35°C, but are not viable at temperatures below 32°C. The effect of the mutation on the action of λ pN (at 40°C) is very different from that of *nus*A1; only the growth of λ*bio*256*sex* is not supported. λ*bio*256*sex* synthesizes lower levels of an altered *N* gene product (S. Adhya, personal communication; unpublished results, this laboratory), and thus is more dependent upon normal levels of Nus proteins in order to make plaques. The failure of λ*bio*256*sex* to grow at 40°C is due specifically to a failure in pN action; a derivative of λ*bio*256*sex* that also carries the *nin*5 deletion grows in the *nus*A*cs*10 host (see Table 2).

Genetic mapping using phage P1 transduction located the mutation(s) responsible for the Cs and Nus phenotypes of the *nus*A gene. The *nus*A gene cotransduces with *arg*G at a frequency of 95%. In the first cross the donor was *arg*G : : Tn*5* and carried a putative *nus*A*cs* allele. The recipient was wild-type at both the *arg*G and *nus*A loci. Of the

Table 2

Effect of nus*A*cs*10 on pN action: efficiency of plating of phage*

Phage	Bacteria, *nus* allele and prophage			
	K1914 *nus*A*cs*10	K2065 *nus*A*cs*10 λ*imm*21*nus*A^+	K2067 *nus*A*cs*10 λ*imm*21*nus*A1	K3767 nus^+ λ*imm*21*nus*A1
λ	0·6	0·8	0·7	1
λ*bio*256*sex*	10^{-6}	0·5	10^{-6}	1
λ*bio*256*sex nin*5	1·0	1·0	1·0	1

Bacteria were grown overnight in LB maltose, sedimented, and resuspended in 0·5 vol. 0·01 M-$MgSO_4$. The phage were diluted and plated on TCMG plates using the indicated bacteria as lawns. Plates were incubated overnight at 40°C. The titer of phage on each of the lawns was divided by the titer on a lawn of K37 (a nus^+ isogenic parent) to give the efficiency of plating. The *nus*A transducing phages were derived from the hybrid phage λ*imm*21. The *imm*21 region encodes a repressor and *N* gene product (Liedke-Kulke & Kaiser, 1967) different from that of λ (Friedman *et al.*, 1973*b*), and thus the hybrid phage expresses a repressor and pN that will not have any effect on phage with the *imm*λ region.

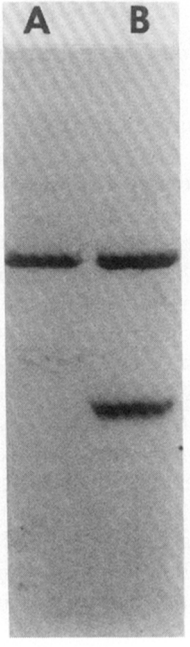

Figure 2. Immuno-electroblot of truncated NusA protein. Extracts of proteins (~10 mg/ml) were made and analyzed by immuno-electroblotting as described in Materials and Methods. Lane A is an extract from *E. coli* strain K37 (24 μg); lane B is an extract from a K37 derivative carrying the pWR324 plasmid (24 μg). Comparison with standards showed the upper band to be in the 69,000 M_r range, while the lower band on the right is about 49,000 M_r. Although NusA protein typically runs at 69,000 M_r on polyacrylamide gels, the molecular weight as determined by DNA sequencing (Ishii *et al.*, 1984; Y. Nakamura, personal communication) is 54,400 M_r. Therefore the 49,000 M_r truncated product may be significantly smaller.

recipients, 93% (138/148) that became resistant to kanamycin (Kan^r) also became Cs and exhibited the mutant NusA phenotype as tested by *λbio256sex* growth. This mapping demonstrates that the mutation(s) responsible for the two phenotypes are either not separable or are extremely closely linked. Although the linkage with *arg*G is completely consistent with an *nus*A mutation(s), it does not rule out the possibility that *nus*A*cs*10 maps to the opposite side of *arg*G.

The results of a second cross localize *nus*A*cs*10 to the *nus*A gene. In this transduction, the donor was once again *arg*G::Tn*5* *nus*A*cs*10, while the recipient carried the *nus*A1 mutation. The *nus*A1 and *nus*A*cs*10 alleles can be distinguished easily by the severity of the Nus$^-$ phenotype imposed. A host with the *nus*A1 mutation fails to support growth of λ wild-type as well as *λbio256sex* at 42°C, while a host with the *nus*A*cs*10 mutation supports growth of the former, but not the latter phage. In this cross, we found that the acquisition of the Cs phenotype is always accompanied by the appearance of the less restrictive Nus phenotype and the loss of the more restrictive NusA1 phenotype; 90/93 Kan^r transductants became Cs and also exhibited the less restrictive phenotype, while 3/93 were cold-resistant and more restrictive. Therefore, the *nus*A*cs*10 mutation is in the same gene as *nus*A1.

A complementation experiment was consistent with the recombination analysis. We used *λimm21nus*A transducing phages containing 12×10^3 base-pairs of DNA from a region of the *E. coli* genome that surrounds *nus*A (S. Adhya, personal communication). The results of this analysis are presented in Table 2. Lysogens of the *nus*A*cs*10 host carrying a *λimm21nus*A$^+$ prophage support *λbio256sex* growth, while those carrying a *λimm21nus*A1 prophage fail to support growth of *λbio256sex* at 42°C.

We also found that a truncated NusA protein complements *nus*A*cs*10 both for growth at 30°C and for λ pN action (data not shown). We transformed a plasmid (pWR324) into the *nus*A*cs*10 and *nus*A1 hosts. The pWR324 plasmid, constructed by L. S. Baron and D. Kopecko (unpublished results), is a pBR322 derivative containing a *nus*A *Pst*I fragment that includes the *nus*A promoter and the 5′ two-thirds of the *nus*A gene. The nature of the NusA protein expressed by the pWR324 plasmid is shown in the immuno-electroblot in Figure 2. Using antibodies prepared against purified *E. coli* NusA protein as the probe, it can be seen that, in addition to the normal NusA protein, a new cross-reacting protein of approximately 49,000 M_r is synthesized in the host containing the pWR324 plasmid. All λ derivatives tested (with the exception of *λbio256sex*) grow well at 40°C on a *nus*A1 host carrying pWR324 (data not shown).

(b) *Overproduction of NusA^{Sal}*

Two lines of evidence led to the idea that NusA^{Sal} protein requires a thymine triplet in *box*A (Friedman & Olson, 1983). First, lambdoid phages that normally can function with NusA^{Sal} (P22 and 21) have *box*A sequences with three T residues (e.g. TGCTCTTT in 21 *nut*R, CGCTCTTT in P22 *nut*L). Second, λ mutants that grow on the *nus*A^{Sal} hybrid bacterium have three T residues at the 3′ end of the *box*A sequence in *nut*R. Conditions were designed to test for NusA^{Sal} activity in the presence of the normal λ *box*A sequence. First, we used a λ derivative that carries *box*A$^+$ and the *pun*A1 and 133 *N* mutations. The mutations in *N* convert the pN into one that functions with NusA^{Sal} protein. Therefore, the failure of *λpun*A1,133 to grow in the NusA^{Sal} host is due solely to the presence of the wild-type *box*A sequence in the *nut*R region. Second, a host carrying *nus*A^{Sal} both on the chromosome and on a plasmid (pNAS1000) was constructed. Because the plasmid carrying the *nus*A^{Sal} gene is multi-copy, we assumed that it would overproduce NusA^{Sal}. Figure 3 shows that this assumption was correct. The proteins electroblotted onto the nitrocellulose paper were probed with antibodies prepared against purified *E. coli* NusA protein. Because equal amounts of proteins

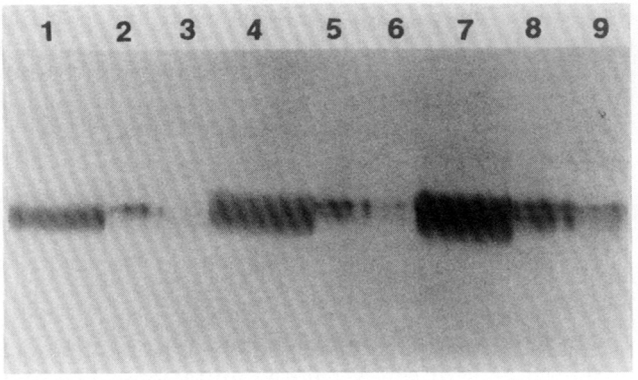

Figure 3. Immuno-electroblot of NusA^{Sal} protein made by pNAS1000. Preparation of protein extracts, electrophoresis, immunoblotting and antigen identification were performed as outlined in Materials and Methods. Samples of each fraction containing 4, 20 or 100 μg were run in adjacent lanes and probed with antibodies raised against *E. coli* NusA protein. Lanes 1 to 3 are dilutions of an extract from K37; lanes 4 to 6 are K1102; lanes 7 to 9 are K1102/pNAS1000.

were loaded onto the gel, the fact that the intensity of the plasmid-carrying strain is greater shows that there is more NusA present in the plasmid-containing strain.

The effect on λ growth of NusA^{Sal} overproduction was determined by measuring the burst of phage following infection. As shown in Table 3, the burst of λ*pun*A1,133 is tenfold greater in the pNAS1000 plasmid-containing NusA^{Sal} host than in the same host without the plasmid.

(c) *Overproduction of pN*

The cloning of the λ *N* gene on a high copy-number plasmid allowed us to address the question of pN specificity. For these experiments, we transferred the wild-type *N* gene to two plasmid vectors. A pBR322 derivative, pUC9 (Vieira & Messing, 1982), was chosen as a high-copy expression vector and the *lac* promoter–*N* gene constructs were transferred to pDPT429, a single-copy R-factor derivative (D. Taylor, unpublished results).

As shown in Table 4, both the single and the multi-copy recombinant plasmids complement λ*N*$^-$ phage for growth. The complementation is specific for *N*: there is no complementation of λ derivatives carrying nonsense mutations in other genes. When *N* is expressed from the unit-copy plasmid λ*N*$^-$ phages are not fully complemented when compared with the effect seen in the case of pN expressed from a single-copy defective λ prophage. This most likely reflects a difference in promoter strength; the plasmid-borne *lac* promoter is weak relative to the prophage *p*L promoter (McClure *et al.*, 1982). Overexpression of *N* by the high-copy plasmid did not have any effect on growth rate when compared

Table 3
Growth of λ in the presence of high levels of NusA^{Sal}: burst size

Phage	Strain, *nus* allele and plasmid: K37 *nusA*$^+$ (*E. coli*)	K1102 *nusA*^{Sal}	K3223 *nusA*^{Sal}/ pNAS1000
λ	422	1	1
λ*pun*A1,133	228	17	290
λ*pun*A1,133 *box*A1	165	283	454

pNAS1000 is a plasmid with the *S. typhimurium nus*A gene cloned into the *tet* gene of pACYC184. Bacteria were grown in LB maltose to ~10^8/ml, sedimented and resuspended in 0·5 vol. 0·01 M-$MgSO_4$. Phage were added to 0·1 ml of bacteria at a multiplicity of 0·1 and adsorbed for 20 min at room temperature. Infected bacteria were diluted into LB containing 10 mM-$MgSO_4$ and incubated at 37°C. Samples were removed at zero time, treated with $CHCl_3$ and titered on K37 (*nus*$^+$) bacteria to give the total number of phage adsorbed. The remaining bacteria were treated with $CHCl_3$ 120 min after infection and titered. The burst is the total number of phage released divided by the total number of infected bacteria. λ*N*$^+$*box*A1 did not give a burst when grown on K3223.

with bacteria carrying pUC9 alone (data not shown).

Although earlier work had shown that λ*N*$^-$ could grow when supplied with the pN of phage P22 (gene *24* product), there was no indication that P22 could function with λ pN (Hilliker & Botstein, 1976; Hilliker *et al.*, 1978). Using a su° host containing the pN-overproducing plasmid pNAS150 as a bacterial lawn, we find that when *N* expression is maximized by adding isopropyl-β-D-thiogalactoside (IPTG) (fully to induce the *lac* promoter, which controls *N* expression), a λ–P22 hybrid with an amber mutation in its *N* gene (*24*am*S4*; Hilliker &

Table 4
Complementation of λN$^-$ *and P22 24*$^-$ *by plasmid clones of λ N*

Phage	Host and plasmid: K37 None	K37 pNAS150	K37 pNAS200	K2166 None
λ	+	+	+	+
λ*N*am7	−	+	−/+	+
λ*imm*P22	+	+	+	+
λ*imm*P22,*24*am*S4*	−	+	−	−

Results are expressed as the presence or absence of plaques on either K37 (*nus*$^+$) or K2166 (λ*c*I857(BAM)$_\Delta$*N*$^+$(H1)$_\Delta$, *nus*$^+$) containing the indicated plasmids at 40°C in the presence of 50 μM-isopropyl-β-D-thiogalactoside. K2166 provides constitutive expression of N protein from a defective prophage at 40°C. pNAS150 is a very high copy plasmid (a pUC9 derivative) with *N*$^+$ under *lac* promoter control; *N*$^+$ is also under *lac* promoter control in pNAS200, but this vector (pDPT429) exists in only 1 to 2 copies per cell. Phage derivatives with nonsense mutations in other essential genes (λ*A*am11, λ*R*am5, λ*Q*am73,501 and λ*imm*P22 2am*N*11) did not form plaques on the hosts listed in this Table, although they did grow on nonsense-suppressing bacteria. Plasmid constructions are described in detail in Materials and Methods.

Botstein, 1976) forms normal plaques (Table 4). Plaque formation is dependent upon the presence of IPTG, indicating that maximum *lac* promoter activity is required.

(d) *Structure of* λpun*A1,133* N *gene protein*

Mapping of the *pun*A133 mutation placed it in or near the *N* gene (Friedman & Olson, 1983). To determine the precise change(s) that enables λ to function with NusA^{Sal}, we sequenced the *N* gene from the λ*pun*A1,133 phage. Fragments containing the *N* genes were cloned from both the mutant phage and the wild-type parent. In each case, the *N* gene was cloned without the associated *nut*L region so that transcripts that initiate at the upstream *lac* promoter in the M13mp9 cloning vector would not have the potential to antiterminate, and possibly to interfere with plasmid replication.

The sequence we determined for the wild-type *N* gene agrees completely with the published sequence (Fig. 4; Franklin & Bennett, 1979; Sanger *et al.*, 1983). The sequence for the *N* from the mutant phage shows only two changes from wild-type, with resulting amino acid changes: (1) the serine to arginine substitution that had previously been determined for the *pun*A1 mutation at nucleotide 125 of the *N* gene (Franklin, 1985); and (2) a lysine to arginine replacement five codons promoter-proximal to *pun*A1 (see Fig. 3). This change, unlike *pun*A1, does not alter the overall charge of the very basic N protein.

A reconstruction experiment demonstrated that only these mutations are required, along the *box*A1 mutation, to permit phage λ growth in a NusA^{Sal} host. A λ carrying *box*A1 and a wild-type *N* (described below) was crossed with a plasmid carrying the mutant *pun*A1,133 *N* gene (pNAS110) or, as a control, a wild-type *N* gene (pNAS100). Only the cross with the *N* from λ*pun*A1,133 yielded recombinants that could grow in the NusA^{Sal} host.

(e) *Phenotype of* λN$^+$box*A1*

To extent our studies on the role of *box*A in pN action, we constructed two derivatives of λ that carry *box*A1 but have a wild-type *N* gene. This permitted direct observation of the effects of *box*A1 in the absence of the *N* (*pun*A) mutations. The first phage is essentially λ wild-type except for the *box*A1 mutation. The second has an r32-IS2 insertion (see Fig. 1) in addition to *box*A1 (see Materials and Methods for details of the constructions). The effect of the *box*A1 mutation on pN action was assessed by measuring the efficiency of plating on *E. coli* mutants. Phage growth provides a good measure of the effect of changes in the *nut*R region (including *box*A), because all phage products that are normally essential for lytic growth, except pN itself, are dependent upon antitermination of transcription initiated at *p*R.

As shown in Table 5, the *box*A1 mutation permits λ to grow in *E. coli* hosts carrying either the *nus*A1 or the *nus*E71 mutation at 42°C. On the other hand, *box*A1 does not markedly improve the growth of λ in other *nus* mutants, such as *nus*B5 (data not shown). The observation that *box*A1 also improves λ growth in the *nus*E71 host is not surprising insofar as a number of phage and bacterial mutants selected for improving λ growth in one mutant host (either *nus*A1 or *nus*E71) also enhance λ growth in

Phage λ N gene

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ATG GAT GCA CAA ACA CGC CGC CGC GAA CGT CGC GCA GAG AAA CAG GCT CAA TGG AAA  57
Met Asp Ala Gln Thr Arg Arg Arg Glu Arg Arg Ala Glu Lys Gln Ala Gln Trp Lys


GCA GCA AAT CCC CTG TTG GTT GGG GTA AGC GCA AAA CCA GTT AAC CTC CCT ATT CTC  114
Ala Ala Asn Pro Leu Leu Val Gly Val Ser Ala Lys Pro Val Asn Leu Pro Ile Leu

                          punA133              punA1
                             G                    A
TCG CTG AAT CGC AAA CCG AAA TCA CGA GTA GAA AGC GCA CTA AAT CCG ATA GAC CTT  171
Ser Leu Asn Arg Lys Pro Lys Ser Arg Val Glu Ser Ala Leu Asn Pro Ile Asp Leu
                          Arg                  Arg

ACA GTG CTG GCT GAA TAC CAC AAA CAG ATT GAA AGC AAC CTG CAA CGT ATT GAG CGC  228
Thr Val Leu Ala Glu Tyr His Lys Gln Ile Glu Ser Asn Leu Gln Arg Ile Glu Arg


AAG AAT CAG CGC ACA TGG TAC AGC AAG CCT GGC GAA CGC GGC ATA ACA TGC AGT GGA  285
Lys Asn Gln Arg Thr Trp Tyr Ser Lys Pro Gly Glu Arg Gly Ile Thr Cys Ser Gly


CGC CAG AAA ATT AAG GGA AAA TCG ATT CCT CTT ATC TAG  324
Arg Gln Lys Ile Lys Gly Lys Ser Ile Pro Leu Ile ---
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Figure 4. DNA and protein sequences of the λ *N* gene showing the *pun*A1 and *pun*A133 mutations. The *pun*A1 mutation changes the serine to arginine; *pun*A133 converts the lysine to arginine.

Table 5
Effect of box*A1 mutation on growth of λ in* E. coli *with mutant* nus *genes: efficiency of plating of phage*

Phage	Strain and *nus* allele		
	K37 *nus*$^+$	K95 *nus*A1	K556 *nus*E71
λ	1	$<10^{-8}$	$<10^{-8}$
λr32	1	$<10^{-8}$	$<10^{-8}$
λ*pun*A1,133	1	1	1
λ*pun*A1,133 r32	1	0·6	$<10^{-4}$
λ*pun*A1,133 *box*A1	1	1	1
λ*pun*A1,133 *box*A1 r32	1	1	1
λ*box*A1	1	0·8	0·5
λ*box*A1 r32	1	1·2	$<10^{-4}$
λ*pun*A1	1	1	1
λ*pun*A1 r32	1	0·6	$<10^{-4}$

Bacteria were grown overnight in LB maltose. Dilutions of phage were titered on the indicated bacterial lawns on TCMG plates and incubated at 42 °C. Efficiency of plating is calculated by dividing the titer of phage observed on the indicated lawn by the titer on K37 (*nus*$^+$) bacteria.

the other *nus*$^-$ host (Friedman *et al.*, 1981; Ward *et al.*, 1983; Schauer, 1985).

A difference in the effect of *box*A1 on the growth of λ in either a *nus*A1 or *nus*E71 mutant is readily observed when the phage carries the r32-IS*2* insertion (Brachet *et al.*, 1970; Tomich & Friedman, 1977) in addition to *box*A1. IS*2* contains a strong ρ-dependent termination signal (de Crombrugghe *et al.*, 1973) and thus is likely to impose a more stringent demand for pN activity at *nut*R. Table 5 shows that the *box*A1 r32 phage grows relatively well in the *nus*A1 host K95, but poorly in the *nus*E71 host K556. Although λ*box*A1 grows on either the *nus*A1 or the *nus*E71 host at all temperatures, it does not grow in a *nus*A1-*nus*E71 double mutant, even at temperatures that are generally more permissive for λ growth (30 to 32 °C; data not shown).

4. Discussion

The *N* transcription antitermination reaction of phage λ is one of the best characterized examples of this type of gene regulation. In spite of this, it is obvious that our knowledge of the system is rudimentary. The primary question at this time is how the phage and bacterial proteins interact with each other, as well as with the *nut* region sequences in the phage nucleic acid. The complexity of the *N* system has prevented the reconstruction of the reaction in a purified system *in vitro*, and therefore genetic analyses of host and phage mutations that influence *N* action have provided significant insights into the antitermination mechanism.

(a) *NusA is essential for* E. coli *growth*

The isolation of a conditionally lethal mutation in the *nus*A gene demonstrates that NusA protein serves an essential role in *E. coli* growth. Our work with the *nus*A*cs*10 mutation complements the studies of Nakamura & Mizusawa (1985), who showed that NusA is required for cell growth at high temperatures. There is reason to suspect that λ may require different levels of NusA protein at high and low temperatures. In particular are observations that: (1) many *nus* mutations impose more severe restrictions on pN action at higher temperatures than at lower temperatures (Friedman & Gottesman, 1983); and (2) *box*A seems to be necessary for pN antitermination in certain plasmid constructs at high, but not low, temperatures (Peltz *et al.*, 1985).

The specific defect caused by the *nus*A*cs*10 mutation has not yet been determined. However, we have isolated second-site suppressors of *nus*A*cs*10 and characterization of these mutations should prove to be useful in determining the nature of the NusA defect in the conditionally lethal mutants. We have used both recombinational mapping and complementation studies to demonstrate that *nus*A*cs*10 and *nus*A1 are alleles of the same gene. The fact that extensive genetic crosses failed to break the linkage between the Cs and Nus phenotypes of *nus*A*cs*10 indicates that either the same or extremely closely linked mutations are responsible for the two phenotypes.

The truncated NusA protein (49,000 M_r) expressed by the pWR324 plasmid complements the *nus*A*cs*10 mutant for growth at low temperature and restores growth of λ*bio*256*sex* (data not shown). Preliminary results indicate that a similarly truncated *nus*A^{Sal} gene product can also complement *nus*A*cs*10 for cell growth (A. T. Schauer & D. I. Friedman, unpublished results).

These findings seem to be in conflict with the results of Nakamura & Mizusawa (1985), who report that the 49,000 M_r NusA protein is not effective in permitting growth of an *E. coli nus*A*ts* mutant at the non-permissive temperature. Although we did not use their plasmid, our vector, insert and cloning scheme were the same and so we have no reason to suspect that there was any difference in the truncated protein being expressed. Perhaps the protein made in the *nus*A*ts* mutant is dominant in the presence of the truncated protein, while the cold-sensitive NusA protein in our mutant is recessive. Alternatively, as discussed above, there may be a different requirement for NusA at high and low temperatures. Another difference between the *nus*A*ts* and *nus*A*cs* alleles is that the cold-sensitive mutation confers a *nus* phenotype, albeit a weak one.

Our experiments with the 49,000 M_r NusA protein suggest that the biological activity of NusA that is required both for λ *N* action and for bacterial viability (at least at lower temperatures) is located in the amino two-thirds of the molecule. The immuno-electroblotting analysis confirms that the pWR234 plasmid expresses a smaller NusA protein. These results mean that the essential recognition sites for other macromolecules on the

NusA protein are likely to be located in the amino two-thirds of the protein. However, we cannot exclude the possibility that carboxy-terminal activity is provided to the truncated protein by the presence of the NusA1 or NusAcs10 proteins in these merodiploids.

In comparing the activities of the NusA products from *E. coli* and *S. typhimurium*, only the difference in the ability to facilitate the action of λ pN appears to be significantly different; NusA^{Sal} can function in *E. coli* growth and support the action of the pN-analogs of lambdoid phages 21 and P22. Our sequence analysis of the *Salmonella nus*A gene and comparison with *E. coli nus*A identifies three segments of heterogeneity between the amino portions of the two proteins (unpublished results). We suggest that these domains may be important sites for these interactions. Computer analysis fails to uncover any significant conformational difference in the structure of the two proteins. Therefore, it is likely that these amino acid heterogeneities represent actual sites of interaction rather than sites that alter secondary or tertiary protein structure (which might lead to abortive interactions at other sites).

(b) λ *pN interacts with P22* nut*R*

It has been suggested that the recognition element for pN in the *nut* region is the sequence of dyad symmetry (Salstrom & Szybalski, 1978). The isolation by Salstrom & Szybalski of the *nut*L mutation in the center region of the dyad demonstrates the importance of this sequence or structure to the *N* reaction. The reason for suspecting that the dyad symmetry may be a target for pN (Friedman & Gottesman, 1983) was based primarily on two observations: first, λ pN appeared to be specific for its own *nut* region, e.g. λ cannot complement a phage 21 N^- (Friedman *et al.*, 1973*b*) or P22 *24*$^-$ phage (Hilliker & Botstein, 1976); and second, there are significant differences in the *nut* dyad symmetries from the different phages which could explain *nut*–pN specificity for the different lambdoid phages.

The observation reported in this paper, that λ pN can complement a P22 N^- (gene *24*) mutant shows that an argument for strict exclusivity in pN action cannot be true. In fact, the earlier work of Hilliker & Botstein (1976) showing that P22 pN can substitute for λ pN elegantly made this argument for the *24* gene product of P22. These observations do not rule out the regions of dyad symmetry as recognition sites for the different *N* products. There is clearly more efficient antitermination activity when a *nut* site interacts with its cognate pN. In addition, as pointed out by J. Greenblatt (personal communication), there are conserved sequences within the different *nut* dyad symmetries which could explain the partial activity of pN proteins for heterologous *nut* sequences.

(c) box*A1 enhances* λ *growth in the* nus*A1 host*

Although there are *nut* regions downstream from both *p*L and *p*R, the *nut* region downstream from *p*R has primary influence over expression of genes that play essential roles in lytic phage growth. This explains why a change in the *box*A sequence of *nut*L (e.g. *box*A1) is not necessary to facilitate λ growth in a NusA^{Sal} host. We have separated the *nut*R region *box*A1 mutation from the *N* (*pun*) mutations. Although the *box*A1 mutation was selected to enhance λ growth with the *Salmonella* NusA protein, it also facilitates λ growth in the *E. coli nus*A1 host, even in the absence of the *N* (*pun*) mutations (Table 5). This indicates that the *box*A1 mutation does not specifically enhance the ability of NusA^{Sal} to operate with λ pN, but rather suggests that it is a change resulting in a more effective *box*A sequence.

The fact that λN^+*box*A1 grows on the *nus*E71 host as well as *nus*A1 (but not *nus*B5 or *nus*C60) calls into question the idea that the *box*A site is specific for NusA protein. However, several lines of evidence support the idea that *box*A1 may improve some type of signal between *box*A and NusA. First, a number of mutations that suppress *nus*A1 for pN action also suppress *nus*E71, including changes in *nus*B as well as in *N* (Ward *et al.*, 1983; Schauer, 1985). Therefore, it might not be surprising that NusA activity facilitated by mutant *box*A could suppress the effect of the *nus*E71 mutation. Second, when a strong ρ-dependent termination signal is placed downstream from the *nut* region (r32-IS*2*; Table 5), the new λ*box*A1 derivative does not form plaques on the *nus*E71 host. Thus, the enhanced growth of λ*box*A1 in the *nus*E71 mutant may merely reflect a more optimal NusA–*box*A interaction. Third, λ*pun*A1, which was selected to grow on the *nus*A1 host, plates equally well on *nus*E71 (Table 5). However, λ*pun*A1 exhibits the same behavior as λ*box*A1 when the r32-IS*2* termination signal is inserted; growth on the *nus*E71 host is abolished. Thus, the stronger terminator prevents λ growth on *nus*E71 in the case of an enhanced pN–NusA interaction in the same manner as in the case of a postulated *box*A1–NusA recognition.

Additional evidence that three T residues in *box*A are more optimal than two derives from the studies with NusA^{Sal}. Provided that there is *pun*A1,133 mutant pN present, λ*box*A1 will grow well in the NusA^{Sal} host. However, even in the presence of the *pun*A1,133 pN, λ with a wild-type *box*A sequence grows very poorly. But if NusA^{Sal} protein is supplied in excess (with pNAS1000), λ*pun*A1,133 *box*A$^+$ will also grow. We suggest that the ability of NusA^{Sal} to function with the wild-type *box*A means that the three T residues in *box*A1 (CGCTCTTT) do not represent a qualitatively different *box*A, but rather a more optimal *box*A sequence for NusA^{Sal}. Thus, the experiments with both the mutant *E. coli* NusA and the natural *Salmonella* variant point to the *box*A sequence with three T residues as a generally more optimal site. Moreover, the fact that

increased levels of NusA product enhance the *N* antitermination reaction strongly argues that NusA is specifically required for the *N*-imposed antitermination. This does not rule out a role for NusA in terminating transcription. NusA could be multifunctional, participating in antagonistic reactions depending on the nature of the other participants.

Finally, we wish to stress that although these genetic studies provide additional support for the idea that *box*A might play some role in NusA recognition, we have no direct proof that *box*A is a *binding* site. It is possible that the NusA–*box*A interaction is indirect; we do not exclude the possibility that the recognition events could take place *via* an intermediary, such as another Nus protein. Binding studies carried out by Tsugawa *et al.* (1985) indicate that NusA associates *in vitro* with a site on the RNA immediately upstream from *box*A. It is not unlikely that the nearby *box*A sequence participates in this association. However, the binding sequence reported by the authors is not present in λ *p*L operon.

We thank the following for their help: Drew Granston for many discussions; Dave McGrath for assistance with some experiments; Jack Greenblatt and Bob Landick for purified NusA protein; Rod Nairn and Richard Smith for help with Western gels; Lathe Claflin for preparation of immune serum; Dale Oxender for computer analysis of NusA secondary structure; Mike Savageau for help in designing the selection for the *arg*G::Tn*5* mutant; Phil Youderian for suggesting the protocol for selecting λ*nus*A^{Sal} and for providing the *Salmonella* bank. This work was supported by grants from the National Institutes of Health to D.I.F.

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Edited by M. Gottesman