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Evidence that ribosomal protein S10 itself is a cellular component necessary for transcription antitermination by phage λ N protein

(transcription termination/*Escherichia coli* Nus proteins/gene regulation)

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ABSTRACT **Bacteriophage λ *N* gene product acts to modify host RNA polymerase allowing the formation of a termination-resistant transcription apparatus. Previous studies have demonstrated that the *nusE*71 mutation that has altered the ribosomal protein S10 prevents N action *in vivo*. Using a coupled transcription–translation system, we demonstrate here that purified S10 protein as well as the 30S ribosomal subunit is sufficient to restore N activity in the *nusE* mutant extract, allowing antitermination of Rho-dependent and Rho-independent terminators. This provides direct biochemical evidence that the S10 protein itself is one of the cellular components necessary for the formation of an antitermination apparatus.**

The *N* gene product of bacteriophage λ acts to suppress transcription termination in *Escherichia coli* (1–5). The action of N depends on a recognition site (*nut*) encoded by the phage genome (6–9). It is thought that N acts to modify RNA polymerase at the *nut* site, allowing the formation of a termination-resistant transcription apparatus (2, 3). This modification process involves several cellular proteins, defined by *nus* mutations (10–14). Two of these proteins—namely, NusA and NusB—have been shown to be essential for N activity *in vitro* (15, 16). The third cellular component is NusE, originally defined by a recessive mutation, *nusE*71 (13). The *nusE*71 mutation has been mapped in the *rpsJ* gene (13), encoding the ribosomal protein S10 (17). The S10 protein of the *nusE* mutant ribosome shows an alteration in net charge (13). Two specific hypotheses have generally been considered to explain how the *nusE* mutation might cause a defect in antitermination. First, the *nusE* mutation might alter a ribosome function to specifically affect the expression of N or Nus factors. Second, antitermination may involve an interaction of the S10 protein (in soluble or ribosome-bound form) with N and Nus factors, and the *nusE* mutation might specifically alter such an interaction.

Using an S30 coupled transcription translation system, we recently developed complementation assays for NusA, NusB, and NusE components (15). We have demonstrated that the *nusE* mutation does not reduce the synthesis of active N, NusA, and NusB proteins (15). Here we demonstrate that purified S10 protein is sufficient to restore antitermination in the *nusE* mutant S30. In support of the second hypothesis considered above, our results provide direct *in vitro* evidence that the S10 protein itself is one of the cellular components involved in transcription antitermination.

MATERIALS AND METHODS

Plasmids and Strains. *Plasmids.* All plasmid DNA templates used in this study (see Fig. 1) were derived from pBR322 as described (15, 18). The N^+ plasmid pKW1 has been described (15). The plasmid pLS10-2, containing *pL–nutL–rpsJ* fusion was constructed as follows: A 1.2-kilobase *Eco*RI fragment containing the P_{S10}-*rpsJ* region was isolated from pLL36 (19) and then inserted at the *Eco*RI site of the expression vector pRK16-F (kindly provided by K. Abremski), containing λ*oL pL nutL* upstream of the *Eco*RI site.

Strains. The *nus*$^+$ and the *nusE*71 strains AD7070 and AD7073, containing a deletion of the entire *gal* operon, have been described (15). AD7068 (λ *c*I857 N^+ *Gal*K$^-$), used to induce S10 production from plasmid pLS10-2, has been described (9).

Extracts, Ribosomal Fractions, and Proteins. *Extracts.* Preparation of S30 and S100 extracts were as described (15).

Ribosomal fractions. S30 extracts treated with 0.5 M potassium acetate (30 min at 0°C) were centrifuged at 150,000 × *g* for 2 hr. Upon removal of the supernatant, a brown material above the clear ribosomal pellet was rinsed out with AKM buffer (10 mM Tris acetate/14 mM magnesium acetate/60 mM potassium acetate/1 mM dithiothreitol, pH 8.2). To obtain high-salt-washed ribosomes, the clear pellet was thoroughly resuspended in AKM buffer containing 1 M potassium acetate (to the original volume of S30), left on ice for 16–20 hr, and centrifuged at 150,000 × *g* for 2 hr. The clear pellet was resuspended into AKM buffer containing 0.3 mM magnesium acetate (to one-fifth original S30 volume). Upon centrifugation at 30,000 × *g* for 1 hr to remove particles, clear supernatant was removed and stored at −70°C.

Ribosomal subunits. To obtain ribosomal subunits, the first ribosomal pellet, prepared from S30 treated with deoxyribonuclease (2 μg/ml), was resuspended in AKM buffer and pelleted twice through 30% sucrose in AKM buffer. The ribosomal pellet was resuspended in AKM buffer containing 0.3 mM magnesium acetate. Upon removal of particles, the supernatant was left in ice for 16 hr and then centrifuged through 10–30% sucrose gradients as detailed in *Results*. Individual fractions of the gradient were dialyzed for 6 hr against AKM buffer and stored at −70°C.

N protein. An N protein fraction was prepared as described (16).

S10 protein. The S10 protein used in this study was kindly provided to us by Lawrence Kahan (Univ. of Wisconsin, Madison). This particular protein preparation, made by William Held (20), was purified from 30S ribosomal proteins by chromatography on two phosphocellulose columns and a Sephadex G-100 column. The S10 protein, also designated P6, is >90% pure (see figure 2 of ref. 20). The protein was stored in 6 M urea/10 mM phosphoric acid (pH adjusted to 6.5 with methylamine)/3 mM mercaptoethanol/0.15 M LiCl at −70°C (20).

S30 Reactions. Conditions for coupled transcription–translation reactions were exactly as described (15). S30

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reactions were carried out in 0.025 ml at 37°C for 15 min and galactokinase assays were done with 1:5 diluted S30 reactions in 0.03 ml at 37°C for 30 min as described (15). One unit of galactokinase is defined as the amount necessary to produce 1 nmol of galactose 1-phosphate in 60 min. Unit/min per ml refers to the time and volume of S30 reactions. Proteins were measured by the method of Lowry *et al.* (21) with bovine serum albumin as a standard.

RESULTS

To measure transcription antitermination *in vitro*, we have employed S30 extracts (30,000 × *g* supernatant) from Δ*gal* strains and carried out coupled transcription–translation reactions programmed with specifically engineered *pL–galK* fusion plasmids (Fig. 1). An N-independent fusion in plasmid pKS107 produces galactokinase in the presence or absence of N protein (15). The N-dependent fusions in plasmids pAD355, pAD348, and pAD3485 produce galactokinase only in the presence of N. This is because the termination signal(s) present between *nutL* and *galK* in these templates reduce

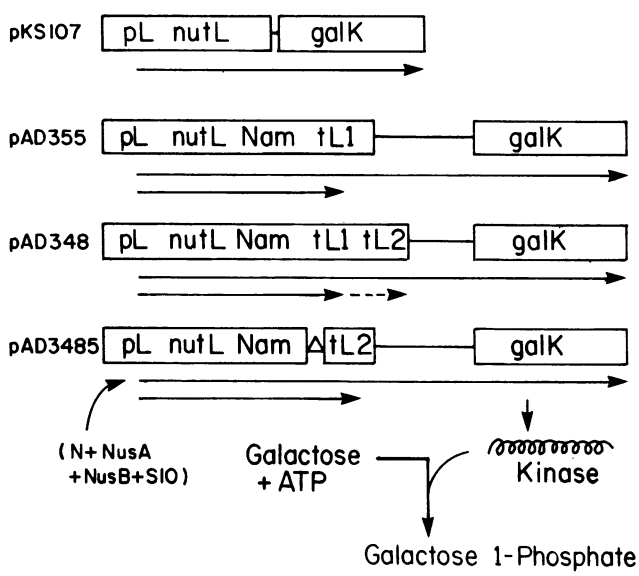

FIG. 1. Plasmid DNA templates with *pL–galK* fusions. Construction of pKS107, pAD355, pAD348, and pAD3485 plasmids and their *in vivo* and *in vitro* properties have been described (15, 18, 22). Only the *pL–galK* fusion part of the respective plasmids is shown in schematic form; the *ori* and *bla* regions are identical in all plasmids. All plasmids are N^- and contain the functional *nutL* site downstream of the *pL* promoter. In pKS107, the *galK* fragment (22) is inserted within the *N* cistron, whereby *N* is fused to *galT* whose translation stops three nucleotides upstream of the initiation signal for *galK* translation (unpublished data). In pAD355, a Rho-dependent terminator is present between *nutL* and *galK* (18). In pAD3485, a Rho-independent terminator is present between *nutL* and *galK* (18). Plasmid pAD348 contains both of these terminators (18) as they are present in the *pL* operon of λ (5). The level of read-through is the same *in vivo* and *in vitro* (14, 18). The higher level of galactokinase produced from pKS107 might reflect the relatively smaller size of *pL–galK* mRNA produced from this plasmid. We do not know whether *galK* translation in pKS107 is enhanced due to translational coupling proposed by Schumperli *et al.* (23); this could account for as much as 50% of the level of galactokinase. It should be noted that the plasmids pAD355, pAD348, and pAD3485 have an identical 1.5-kilobase region upstream of *galK*, which is not present in pKS107. Although this region (pBR322 plasmid sequence) does not contribute to transcription termination (18), transcriptional pause in this region might produce a kinetic difference in the expression of *galK*. In addition to these possibilities, antitermination by N might be incomplete *in vivo* and *in vitro*.

galK transcription; the action of N at *nutL* allows suppression of transcription termination and thereby causes the synthesis of galactokinase at elevated levels (15).

An analysis of NusE-complementing activity is presented in Table 1. A *nus*$^+$ extract produces a high level of galactokinase from plasmid pAD355 only in the presence of N (Table 1, lines 1 and 2). In contrast, the *nusE* extract fails to synthesize significant amounts of galactokinase from pAD355 even in the presence of N (Table 1, lines 3 and 4). The defect of *nusE* extract in *galK* synthesis must be at the level of regulation of transcription termination, since the terminatorless *pL–galK* fusion in pKS107 produces similar amounts of galactokinase in *nus*$^+$ and *nusE* extracts (Table 1, lines 5 and 6). The defect of *nusE* extract can be complemented by the addition of *nus*$^+$ extract: a ribosome-free S100 supernatant as well as the high-salt-washed ribosomal fraction complement the *nusE* extract to restore a high level

Table 1. Analysis of NusE-complementing activity

	Template DNA	S30 extract	N fraction	NusE fraction	Galactokinase, units/min per ml
1.	pAD355	*nus*$^+$	−	—	49
2.	pAD355	*nus*$^+$	+	—	523
3.	pAD355	*nusE*	−	—	43
4.	pAD355	*nusE*	+	—	29
5.	pKS107	*nus*$^+$	−	—	2450
6.	pKS107	*nusE*	−	—	2510
7.	pAD355	*nusE*	+	*nus*$^+$ S100	412
8.	pAD355	*nusE*	+	*nus*$^+$ Rib	550
9.	pAD355	*nusE*	−	*nus*$^+$ S100	56
10.	pAD355	*nusE*	−	*nus*$^+$ Rib	22
11.	pAD355	*nusE*	+	*nusE* S100	36
12.	pAD355	*nusE*	+	*nusE* Rib	10
13.	pAD355	*nusE*	+	S10	756
14.	pAD355	*nusE*	−	S10	37
15.	pKS107	*nusE*	−	S10	2505
16.	pAD355	*nusE*	+	S10*	755
17.	pAD355	*nusE*	+	*nus*$^+$ S100*	384
18.	pAD355	*nusE*	+	*nus*$^+$ Rib*	529
19.	pAD3485	*nusE*	+	—	32
20.	pAD3485	*nusE*	−	S10	94
21.	pAD3485	*nusE*	+	S10	518
22.	pAD348	*nusE*	+	—	39
23.	pAD348	*nusE*	−	S10	88
24.	pAD348	*nusE*	+	S10	379

S30 reactions were carried out with 6.8 mg of S30 extract per ml, 20 μg of DNA per ml, 0.9 mg of S100 or high-salt-washed ribosomal fractions per ml, and 4.8 μg of S10 protein per ml. To add S10, a solution at 1.2 mg/ml was diluted appropriately in 0.5× KUP buffer (0.5 M urea/0.5 M KCl/1 mM potassium phosphate/0.5 mM dithiothreitol, pH 7.0) as described by Baughman and Nomura (24) and added to the S30 reaction (2 μl/25 μl). The same amount of buffer was added in control reaction mixtures and it was found not to affect *galK* expression from pKS107 or from pAD355 in the presence of N to any significant level (≤2%). In lines 16–18, the respective complementing fractions (designated with asterisk) were treated at 0°C with 10 mM *N*-ethylmaleimide for 10 min and then with 30 mM dithiothreitol for 10 min. In the case of S10, this treatment was done in KUP buffer. The S30 reactions were initiated by transferring tubes from 0°C to 37°C and stopped by a 1:5 dilution into S30 stop buffer (1 mM triethanolamine/1 mM dithiothreitol/100 mM Tris·HCl, pH 7.9). The diluted S30 was then used for the galactokinase assay (15). Note that pKS107 reactions were diluted 1:5 and the galactokinase assay was done for 15 min instead of usual 30-min period. This allowed the measurement of the rate of galactokinase synthesis from pKS107. Background values of 20 and 31 units obtained without DNA in *nus*$^+$ and *nusE* extracts, respectively, were subtracted. One unit is defined as the amount of galactokinase necessary to produce 1 nmol of galactose 1-phosphate in 60 min at 37°C. Unit/min per ml refers to the time and volume of the S30 reaction.

synthesis of galactokinase (Table 1, lines 7 and 8); these fractions do not influence galactokinase synthesis in the absence of N (Table 1, lines 9 and 10). Similar fractions prepared from the *nusE* mutant are inactive in elevating galactokinase synthesis (Table 1, lines 11 and 12). We conclude that the *nusE* extract is defective in some specific cellular component necessary for N activity.

The cellular component that is defective (or missing) in the *nusE* mutant extract could be the S10 protein itself or a protein whose expression is somehow affected by the *nusE* mutation. Since the *nusE* extract is capable of complementing a *nusA* or *nusB* extract, and since both *nusA* and *nusB* extracts complement the *nusE* extract to allow N activity, the *nusE* defect cannot be due to a lack of functional NusA and NusB proteins (15). If the *nusE* extract is defective in N activity due to the fact that antitermination requires functional S10 protein, purified 30S ribosomal subunit or the S10 protein itself might be able to restore N activity in this extract. We show below that both 30S subunit and purified S10 protein are capable of specifically complementing the *nusE* defect.

Purified ribosomal fraction was incubated with a low concentration of Mg^{2+} and subsequently fractionated into 50S and 30S subunits by centrifugation through 10–30% sucrose gradients (see Fig. 2*A*). Individual fractions of the gradient were used to complement the *nusE* extract for

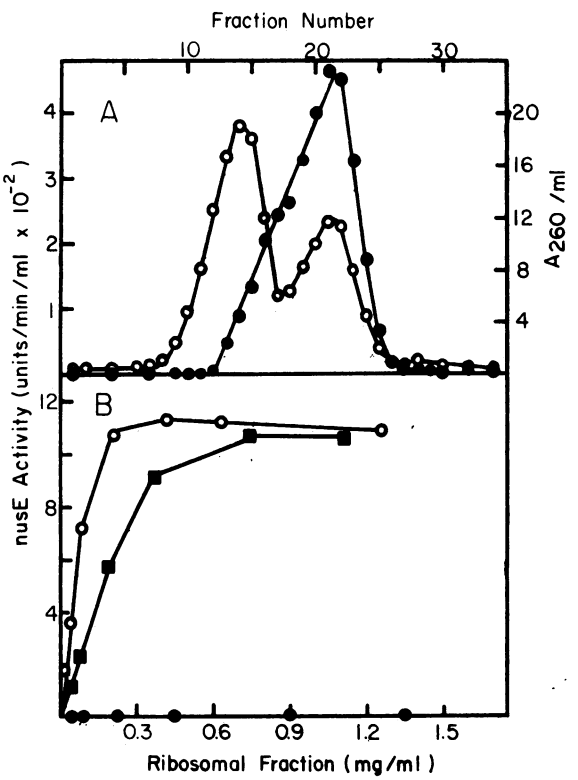

FIG. 2. NusE-complementing activity of 30S subunit. (*A*) 70S ribosome was purified from deoxyribonuclease-treated S30 extract of AD7070 (*nus*$^+$). Subunit dissociation was carried out at a concentration of 300 A_{260} units/ml. Samples of 0.5 ml of ribosomes at 200 A_{260} units/ml were loaded on 12-ml 10–30% sucrose gradients and centrifuged at 100,000 × *g* in an SW41 rotor for 12 hr, and fractions of 13 drops each were collected from the bottom of the tubes. After dialysis against AKM buffer for 6 hr, 4 μl of each fraction was assayed for NusE activity. ●, *nusE* activity; ○, A_{260}. (*B*) Fractions 12 and 22 of the gradient were treated with 0.42 g of ammonium sulfate per ml. Precipitates were collected by centrifugation at 50,000 × *g* for 30 min, dissolved in AKM buffer, and dialyzed against the same buffer. Indicated amounts of these fractions were assayed for NusE activity. ■, 70 S; ●, 50 S; ○, 30 S.

N-stimulated synthesis of galactokinase from plasmid pAD355. The entire NusE-complementing activity of the ribosome fractionated as a single peak associated with the 30S subunit (Fig. 2*A*). Thus, no NusE activity was dissociated from the ribosome during subunit fractionation. Compared to the 30S subunit or the undissociated ribosomal fraction, the 50S subunit showed very little or no stimulatory activity (Fig. 2*B*). These results suggest that the NusE-complementing activity is an integral component of the 30S subunit of the ribosome.

We next tested the effect of purified S10 protein on galactokinase synthesis from pAD355. This 12-kilodalton protein was originally purified by Held *et al.* (20) from the 30S subunit as a component required for the assembly and activity of 30S ribosome. In our complementation assay, S10 was fully functional to allow high-level synthesis of galactokinase in the *nusE* mutant extract (Table 1, line 13). The stimulation of galactokinase synthesis by S10 was N dependent (Table 1, line 14). S10 did not stimulate *galK* expression from the N-independent *pL–galK* fusion in plasmid pKS107 (compare lines 6 and 15 of Table 1). It also did not complement a *nusA* or *nusB* mutant extract to restore N activity (data not shown). Thus, S10 specifically restored N activity in the *nusE* extract.

The following lines of evidence support the conclusion that the S10 protein is both necessary and sufficient to restore antitermination in the *nusE* mutant extract:

(*i*) A multiple-copy plasmid encoding the functional *rpsJ* gene (see *Materials and Methods*) led to ≈10-fold overproduction of the NusE-complementing activity (Fig. 3). If the NusE-complementing activity obtained with the S10 protein preparation was due to a minor contaminant, its expression is not expected to be augmented by the multicopy *rpsJ* plasmid.

(*ii*) The soluble NusE activity of a *nus*$^+$ extract was fractionated and eluted as a single peak of activity from the phosphocellulose column with 0.25 M salt. The purified S10 protein and the plasmid-induced NusE component coeluted with this activity; no other peak of activity was detected (unpublished data).

(*iii*) The activity of S10 protein is resistant to *N*-ethylmaleimide, a sulfhydryl reagent that forms a covalent bond with cysteine residues (Table 1, line 16). The NusE activity associated with the ribosome as well as with S100

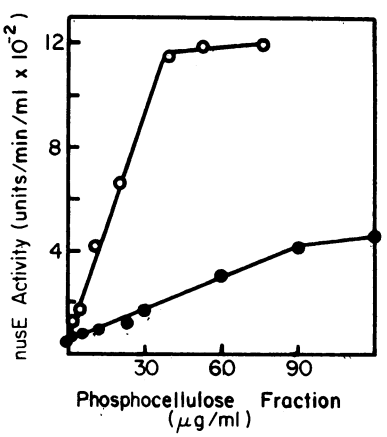

FIG. 3. Overproduction of NusE-complementing activity by multicopy S10 plasmid. AD7068 (pLS10-2) was grown in HPM medium (15) at 32°C to A_{600} = 1.0 and then at 42°C for an additional 2 hr. S100 extracts from this and AD7070 were made and fractionated on two phosphocellulose columns (16). After washing the columns with 0.1 M potassium acetate, the NusE fraction was eluted with 0.5 M potassium acetate. The two phosphocellulose fractions were then compared for NusE-complementing activity as described in Table 1 using pAD355 DNA. ●, AD7070; ○, AD7068 (pLS10-2).

supernatant is entirely resistant to *N*-ethylmaleimide (Table 1, lines 17 and 18). The plasmid-induced NusE activity is also resistant to *N*-ethylmaleimide (data not shown). The lack of a cysteine residue in the primary structure of S10 polypeptide (17) is consistent with these results. Note that N and NusA are sensitive to *N*-ethylmaleimide but NusB is resistant to this compound (16).

The above described experiments showed that S10 is sufficient to allow N suppression of a Rho-dependent terminator. Positive regulation of λ gene expression by N involves suppression of several Rho-dependent and Rho-independent terminators (5, 25). The antitermination of either class of terminators by N involves both NusA and NusB proteins, which are required in approximately equimolar ratio (16, 18). To further examine the role of S10, we have tested its effect on the suppression of single and multiple terminators that vary with respect to Rho dependence (18). Our results show that S10 protein is able to allow N suppression of a Rho-independent terminator in the *nusE* extract (Table 1, lines 19–21). S10 is also sufficient to allow N suppression of multiple terminators located between *nutL* and *galK* (Table 1, lines 22–24). These results demonstrate that the role of S10 in N action is independent of the nature of terminator sequences. The ability of S10 to allow the suppression of multiple terminators suggests that it is a vital component necessary for the formation of a termination-resistant transcription apparatus.

To determine the stoichiometry of S10 protein in relation to NusA and NusB proteins, we have measured galactokinase synthesis from plasmid pAD355 in *nusE* extract in the presence of various amounts of S10 (Fig. 4). Approximately 1.3 μg of S10 per ml was sufficient to obtain half-maximal activity. Our previous results showed that ≈2.7 μg of NusA per ml and 0.6 μg of NusB per ml are sufficient for half-maximal activity with the same template DNA (16). The molecular weights of each of the three proteins have been predicted from the respective DNA sequences (17, 26–28). Assuming that all of these protein preparations contain similar fractions of active molecules, we estimate that NusA, NusB, and S10 are required in a 1:1:2 ratio. This suggests that approximately stoichiometric amounts of these three cellular components participate in the formation of the antitermination apparatus.

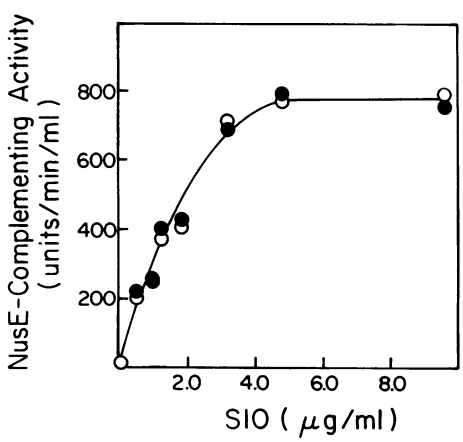

FIG. 4. Stoichiometry of S10. Plasmid pAD355 was used to measure N activity in the *nusE* extract in the presence of various amounts of S10. Duplicate reactions (shown with open and solid circles) with constant volume of differentially diluted S10 protein were carried out. Dilution of S10 was done as described in the legend to Table 1. A background level of 35 units of galactokinase, obtained without S10, was subtracted.

DISCUSSION

Results presented here provide direct biochemical evidence that the ribosomal protein S10 is one of the cellular components required for transcription antitermination mediated by the N protein of phage λ. The role of S10 in antitermination is independent of the nature of terminator sequences, and it is required for the suppression of multiple terminators located downstream of a *nut* site. Thus, S10, like NusA and NusB, is involved in the formation of an antitermination apparatus. The fact that these three cellular proteins are required in roughly stoichiometric amounts is consistent with the hypothesis that S10 protein might serve as a subunit of the proposed antitermination apparatus. This would be a situation analogous to the role of the ribosomal protein S1, which acts as a subunit of the phage Qβ RNA replicase (29).

There are two possible forms with which S10 could participate in antitermination: (*i*) as a soluble protein without involving ribosomes and (*ii*) as a component of the ribosome with or without an involvement of translation. Purified S10 protein can complement the *nusE* defect, implying that S10 might act as a soluble protein. Conversely, the 30S subunit also complements the *nusE* defect, implying that S10 might be acting as a part of the ribosome. Two models can account for these results:

(*i*) Free S10 and ribosome-bound S10 may be equally functional in N action. This assumes that the ribosome may not play a necessary role and, in addition, does not sterically hinder the interaction of S10 with the antitermination apparatus. Although S10 is an integral component of the 30S subunit, parts of S10 lie at the surface of the subunit, as demonstrated by its reaction with antibody (30, 31).

(*ii*) If under our reaction condition there is an appreciable exchange between soluble and ribosome-bound S10, both fractions would be able to contribute functional S10 for N activity. It has been suggested that only 70% of the ribosomes contain S10 (32, 33). Moreover, S10 is one of the few 30S surface proteins whose assembly into the subparticle *in vitro* occurs rapidly at 37°C (20, 34). Thus, S10 could bind to those 30% ribosomes that were devoid of this protein. Such binding of surface proteins has been documented and shown to enhance ribosomal activity (32, 33).

A possible involvement of ribosome and translation in antitermination was considered earlier by Adhya and coworkers (35), who suggested that N could prevent ribosome discharge from the nascent mRNA to inhibit transcription termination. Several lines of evidence have since then argued against the role of translation and translational coupling in N action. (*i*) Ishii *et al.* (36) have demonstrated N activity in S100 extracts in the absence of translation. It is, however, not clear whether the observed N activity is optimal and whether the S100 extract is free of ribosomes. (*ii*) By gene fusion analysis, Warren and Das (9) have demonstrated that translation upstream of the *nut* site does not play a necessary role in N action *in vivo*. This provided evidence against the translational coupling model formally presented by Ward and Gottesman (37).

However, these results do not rule out the participation of ribosomes without the involvement of translation. Consistent with the role of other ribosomal proteins in N activity, A. Schauer and D. Friedman (personal communication) have isolated a suppressor of the *nusE71* mutation that is located in the *rplP*, encoding the L16 protein. Let us consider whether a complex of ribosome and RNA polymerase could be made at the *nut* site without the involvement of translational coupling. A compensatory N mutation, *punA*, suppresses both *nusA* and *nusE* mutations; this led Friedman and co-workers to suggest that S10 and N might interact (13). By complementation analysis, N is found to be tightly associated

with the ribosome (15), suggesting that an N–ribosome complex may be formed. The fact that translation termination in close proximity to the *nut* site prevents N activity indicates that the *nut* site RNA is the target of N action (9). Assuming that the N protein interacts specifically with the *nut* site RNA as well as the ribosome, the N–ribosome complex may bind to the *nut* site RNA, leading to the formation of a ribosome–RNA polymerase complex.

In conclusion, N-mediated transcription antitermination involves at least three cellular proteins—NusA, NusB, and S10. Additional cellular components might be involved in N activity. These include a 25-kilodalton protein that binds to N (38) and the "U" protein (24, 39), whose genetic alteration suppresses both *nusA* and *nusE* mutations (S. Sullivan and M. Gottesman, personal communication). Whether the ribosome itself is involved remains an open question.

Note Added in Proof. Since submission of this manuscript, we have obtained further evidence that the S10 protein is both necessary and sufficient to restore N activity in the *nusE* mutant extract. First, an N^+ *nusE* mutant extract that by itself fails to support antitermination is fully active when supplemented with the pure S10 protein. This shows that the *nusE* mutant extract did not lack any component other than the functional S10 protein that might be necessary for N activity. Second, a nearly homogenous preparation of the N protein (kindly provided by William Whalen) allowed antitermination when the N^- *nusE* mutant extract was supplemented with the S10 protein (905 units of galactokinase, as compared with 756 units obtained with the partially purified N protein fraction shown in Table 1, line 13). This shows that the N protein fraction used throughout this study did not provide additional factors that the *nusE* mutant extract might have lacked. Thus, the defect of the *nusE* mutant in antitermination is a direct consequence only of the alteration in the S10 protein rather than of any secondary effects on gene expression. In support of our hypothesis that N might specifically interact with the ribosome to allow antitermination, we have recently observed that the *nusE* mutation has affected the binding of N protein with the 30S ribosomal subunit (unpublished results).

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