Identification of regulatory targets for the bacterial Nus factor complex

Gabriele Baniulyte\textsuperscript{1,2}, Navjot Singh\textsuperscript{1}, Courtney Benoit\textsuperscript{1}, Richard Johnson\textsuperscript{1,2}, Robert Ferguson\textsuperscript{1}, Mauricio Paramo\textsuperscript{1}, Anne M. Stringer\textsuperscript{1}, Ashley Scott\textsuperscript{1}, Pascal Lapierre\textsuperscript{1}, and Joseph T. Wade\textsuperscript{1,2,3}

\textsuperscript{1}Wadsworth Center, New York State Department of Health, Albany, New York, USA.
\textsuperscript{2}Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, New York, USA.
\textsuperscript{3}Corresponding author: joseph.wade@health.ny.gov
ABSTRACT

Nus factors are broadly conserved across bacterial species, and are often essential for viability. A complex of five Nus factors (NusB, NusE, NusA, NusG and SuhB) is considered to be a dedicated regulator of ribosomal RNA folding, and has been shown to prevent Rho-dependent transcription termination. We have established the first cellular function for the Nus factor complex beyond regulation of ribosomal assembly: repression of the Nus factor-encoding gene, suhB. This repression occurs by translation inhibition followed by Rho-dependent transcription termination. Thus, the Nus factor complex can prevent or promote Rho activity depending on the gene context. Extensive conservation of NusB/E binding sites upstream of nus factor genes suggests that Nus factor autoregulation occurs in many species. Putative NusB/E binding sites are also found upstream of many other genes in diverse species, and we demonstrate Nus factor regulation of one such gene in *Citrobacter koseri*. We conclude that Nus factors have an evolutionarily widespread regulatory function beyond ribosomal RNA, and that they are often autoregulatory.
INTRODUCTION

Nus factors are widely conserved in bacteria and play a variety of important roles in transcription and translation. The Nus factor complex comprises the four classical Nus factors, NusA, NusB, NusE (ribosomal protein S10), NusG, and a recently discovered member, SuhB. As a complex, Nus factors serve an important role in promoting expression of ribosomal RNA (rRNA). A NusB/E complex binds BoxA sequence elements in nascent rRNA, upstream of the 16S and 23S genes. Once bound to BoxA, NusB/E has been proposed to interact with elongating RNAP via the NusE-NusG interaction. The role of NusA in Nus complex function is unclear, but may involve binding of NusA to RNA flanking the BoxA. NusA has also been proposed to be a general Rho antagonist by competing with Rho for RNA sites. Early studies of Nus factors focused on their role in preventing both Rho-dependent and intrinsic termination of \( \lambda \) bacteriophage RNAs ("antitermination"), which is completely dependent on the bacteriophage protein N. Nus factors can prevent Rho-dependent termination in the absence of N, and for many years, Nus factors were believed to prevent Rho-dependent termination of rRNA. However, it was recently shown that rRNA is intrinsically resistant to Rho termination, and that the primary role of Nus factors at rRNA is to promote proper RNA folding during ribosome assembly.

The most recently discovered Nus factor, SuhB, has been proposed to stabilize interactions between the NusB/E-bound BoxA and elongating RNAP, thus contributing to proper folding of rRNA. Genome-wide approaches revealed that \( suhB \) is upregulated in the presence of the Rho inhibitor bicyclomycin, suggesting that \( suhB \) is subject to premature Rho-dependent transcription termination. Surprisingly, \( suhB \) is also one of the most upregulated genes in \( \Delta nusB \) cells, suggesting a possible autoregulatory function for Nus factors. Moreover, autoregulation of \( suhB \) has been suggested previously, although the mechanism for this regulation is unclear. Here, we show that \( suhB \) is translationally repressed by Nus factors, which in turn leads to premature Rho-dependent transcription termination. This represents a novel mechanism for control of premature Rho-dependent termination, and is the first described cellular function for Nus factors beyond regulation of rRNA.
Moreover, the role of Nus factors at suhB is to promote Rho-dependent termination of suhB, in contrast to their established function in antagonizing Rho. Bioinformatic analysis suggests that regulation by Nus factors is widespread, and that autoregulation of suhB, nusE or nusB is a common phenomenon. We confirm Nus factor association with suhB mRNA in Salmonella enterica, and we demonstrate Nus factor regulation of an unrelated gene in Citrobacter koseri. Thus, our data show that Nus factors are important regulators with diverse targets and diverse regulatory mechanisms.
RESULTS

Rho-dependent termination within the suhB gene

Genome-wide analysis of Rho termination events suggested Rho-dependent termination within the *E. coli* suhB gene \(^{13,14}\). To confirm this, we used Chromatin Immunoprecipitation (ChIP) coupled with quantitative PCR (ChIP-qPCR) to determine RNAP association across the suhB gene in wild-type cells and cells expressing a mutant Rho (R66S) that has impaired termination activity, likely due to a defect in RNA loading \(^{16}\). In wild-type cells, we observed a large decrease in RNAP association at the 3’ end of suhB relative to the 5’ end. This decrease was substantially reduced in *rho* mutant cells (Fig. 1). Thus, our ChIP data independently support the observation of Rho termination within suhB \(^{13,14}\).

Nus factors are trans-acting regulators of suhB

Based on an approach used to identify modulators of Rho-dependent termination within *S. enterica* chiP \(^{17}\), we used a genetic selection to isolate 30 independent mutants defective in Rho-dependent termination within suhB (see Methods). All 30 strains isolated had a mutation in one of three genes: *nusB* (14 mutants), *nusE* (13 mutants) or *nusG* (3 mutants) (Table S1). We then measured RNAP association across the suhB gene in wild-type, Δ*nusB* and *nusE* mutant cells (*nusE* A12E mutant isolated from the genetic selection). Mutation of *nusB* or *nusE* increased RNAP binding at the suhB 3’ end ~4-fold compared to wild-type cells (Fig. 1 and S1). We conclude that Nus factors promote Rho-dependent termination within the suhB gene. However, RNAP occupancy at the 3’ end of suhB in *nusB* and *nusE* mutants was substantially lower than in the *rho* mutant (Fig. 1 and S1). This difference may be due to spurious, non-coding transcripts arising from nearby intragenic promoters, which are widespread in *E. coli* \(^{18}\) and are often terminated by Rho \(^{13,14}\).

A functional BoxA in the suhB 5’ UTR
We identified a sequence in the suhB 5’ UTR with striking similarity to boxA sequences from rRNA loci (Fig. S2). Moreover, this boxA-like sequence is broadly conserved across Enterobacteriaceae species (Fig. 2A and S3), suggesting that it is a genuine binding site for NusB/E. We generated a library of randomly mutated suhB-lacZ transcriptional fusions (see Methods), and identified fusions that had higher expression of lacZ. All identified mutants carried a single nucleotide change at one of five different positions within the putative boxA (Fig. 2B). We then constructed a strain carrying two chromosomal point mutations in the putative suhB boxA (C4T/T6C; numbers corresponding to the position in the consensus boxA; Fig. S2). We used ChIP-qPCR to measure association of FLAG-tagged SuhB at the 5’ end of the suhB gene in wild-type cells, or cells containing the boxA mutation. We detected robust association of SuhB-FLAG in wild-type cells, but not in the boxA mutant strain (Fig. 2C). We conclude that the putative BoxA in the 5’ UTR of suhB is genuine, and recruits Nus factors. To test whether the BoxA controls Rho-dependent termination within suhB, we measured RNAP occupancy across suhB in the boxA mutant strain. We detected a ~4-fold increase in RNAP occupancy in the downstream portion of suhB in the boxA mutant strain relative to wild-type cells, mirroring the effect of mutating nusB or nusE (Fig. 1). Our data support a model in which Nus factor recruitment by the suhB BoxA leads to Rho-dependent termination within the gene.

BoxA-mediated translational repression of suhB leads to intragenic Rho-dependent transcription termination

The suhB BoxA is separated by only 6 nt from the Shine-Dalgarno (S-D) sequence (Fig. 2A). Rho cannot terminate transcription of translated RNA, likely because RNAP-bound NusG interacts with ribosome-associated NusE (S10)⁶. Hence, we hypothesized that NusB/E association with BoxA sterically blocks association of the 30S ribosome with the mRNA, repressing translation initiation, uncoupling transcription and translation, and thereby promoting Rho-dependent termination. To test this hypothesis, we used the suhB-lacZ transcriptional fusion (Fig. 3A), as well as an equivalent translational fusion (Fig. 3B). We reasoned that mutation of nusB, nusE, or boxA would result in increased expression from both reporter fusions, since these
mutations would relieve translational repression (reported by the translational fusion), which in turn would reduce Rho-dependent termination (reported by the transcriptional fusion). In contrast, we reasoned that mutation of rho would result in increased expression only from the transcriptional fusion reporter, since the SuhB-LacZ fusion protein (from the translational fusion construct) would still be translationally repressed. We measured expression of lacZ from each of these reporter fusions in wild-type cells, and cells with ΔnusB, nusE A12E, or rho R66S mutations. We also measured expression of lacZ in these strains using reporter fusions carrying the C4T/T6C boxA mutation. Consistent with our model, we detected increased expression of both reporter fusion types in mutants of nusB, nusE or boxA, whereas mutation of rho resulted in increased expression of the transcriptional fusion but not the translational fusion reporter (Fig. 3A-B). Note that mutation of nusB, nusE or boxA does not lead to the same level of increase in expression of the reporter fusions (Fig. 3A-B). This is likely due to the fact that mutations in Nus factors have extensive pleiotropic effects, presumably due to the importance of Nus factors in ribosome assembly. Moreover, mutation of boxA in a nusE mutant leads to a further increase in reporter expression, whereas mutation of boxA in a nusB mutant does not (Fig. 3A-B). This is likely due to the mutant NusE retaining partial function, whereas deletion of nusB completely abolishes Nus factor function.

To confirm the effects of mutating nusB, nusE, rho and boxA on expression of suhB in the native context, we measured SuhB protein levels by Western blotting using strains expressing a C-terminally FLAG-tagged derivative of SuhB. We compared SuhB protein levels in cells with nusE A12E, rho R66S, or boxA C4T/T6C mutations; we have previously shown that SuhB protein levels are increased in a ΔnusB mutant. SuhB protein levels in the mutant strains correlated well with the translational suhB-lacZ fusion reporter gene assay: mutation of nusE or boxA caused a modest increase in SuhB-FLAG levels, whereas mutation of rho had no discernible effect (Fig. 3C-D).
Rho-dependent transcription termination occurs early in the *suhB* gene, and requires a Rho-loading sequence that overlaps the BoxA

Rho-dependent termination requires a Rho loading sequence known as a Rut that typically occurs >60 nt upstream of the termination site(s), is pyrimidine-rich, and G-poor. To localize the Rut and the downstream termination site(s), we constructed a short transcriptional *suhB-lacZ* fusion that includes only the first 57 bp of the *suhB* gene. Expression of this reporter fusion was substantially higher in *rho* mutant cells than in wild-type cells (Fig. 4A). In contrast, expression was only marginally higher in *rho* mutant cells than in wild-type cells when the boxA sequence was mutated (Fig. 4A). Thus, the short *suhB-lacZ* reporter fusion behaves similarly to the fusion that includes the entire *suhB* gene (Fig. 3A), indicating that the rut and termination sequences must be upstream of position 57 within *suhB*.

Given that the short *suhB-lacZ* fusion includes only 94 bp of transcribed sequence from *suhB* and its 5’ UTR, and that Rut sequences are typically found >60 nt from the site(s) of termination, we reasoned that the Rut is likely located close to the 5’ end of the *suhB* 5’ UTR. Consistent with this, positions 2-22 of the 5’ UTR include 17 pyrimidines and only one G. This sequence completely encompasses the boxA, suggesting that the boxA and rut sequences overlap. To determine whether mutation of the boxA affects Rho-dependent termination independent of Nus factor-mediated translational repression, we constructed short *suhB-lacZ* fusions in which the *suhB* start codon was mutated, either alone or in conjunction with a mutated boxA. We reasoned that mutation of the *suhB* start codon would bypass the need for BoxA-mediated translational repression to cause Rho-dependent termination. As expected, expression of the fusion with the mutated start codon but wild-type boxA was substantially higher in a *rho* mutant than in wild-type cells (Fig. 4A), consistent with this construct being Rho-terminated. However, expression of the fusion with the mutated start codon and mutated boxA was only marginally higher in a *rho* mutant than in wild-type cells, indicating that Rho-dependent termination is disrupted by mutation of the boxA, even in the absence of *suhB* translation. We conclude that mutation of the boxA reduces Rho-dependent termination by disrupting the rut. This likely occurs due to the boxA and rut
sequences overlapping, in which case mutating the boxA would also alter the rut. However, mutation of the boxA might also alter RNA secondary structure of the rut.

Mutation of the boxA results in greatly decreased Rho-dependent termination of a fusion of the entire suhB gene to lacZ (Fig. 3A). Although this effect could be due to disruption of a rut overlapping the boxA, we reasoned that there are likely to be additional rut sequences within the suhB ORF. To test this hypothesis, we constructed transcriptional fusions of the entire suhB gene and 5’ UTR to lacZ with a mutation in the suhB start codon, either alone or in conjunction with a mutation in the boxA. For both constructs, expression was substantially higher in a rho mutant than in wild-type cells (Fig. 4B), indicating robust Rho-dependent termination within the suhB gene, even with a mutated boxA. We conclude that the suhB gene includes at least one additional rut, and that the effect of mutating the boxA on Rho-dependent termination with a long transcriptional fusion (Fig. 3) is due to loss of Nus factor binding rather than a direct effect on Rho loading.

**BoxA-mediated occlusion of the S-D sequence is not due to steric occlusion**

The data described above are consistent with a steric occlusion model in which NusB/E binding to the BoxA directly prevent 30S ribosome association with the Shine-Dalgarno sequence. However, other mechanisms of translational repression are also possible. The steric occlusion model predicts that increasing the distance between the boxA and S-D elements would relieve translational repression, and consequently Rho-dependent termination. We constructed suhB-lacZ transcriptional fusions that carried insertions of sizes from 2 to 100 bp between the boxA and S-D sequences (see Methods for details). We constructed equivalent fusions carrying a boxA mutation (C4A; Fig. S2). Surprisingly, separating the BoxA and S-D sequences with up to 100 nt intervening RNA did not abolish BoxA-mediated repression (Fig. 5). Note that differences in absolute expression levels for the different constructs are likely due to variability in secondary structure around the ribosome binding site. Additionally, we are confident that none of the insertions inadvertently introduces a new promoter, since a similar construct lacking an active upstream promoter was only weakly expressed (Fig. S4).
We conclude that the steric occlusion model is insufficient to explain BoxA-mediated translational repression of suhB, although the proximity of the BoxA and S-D sequences suggests that simple occlusion would prevent ribosome binding.

We reasoned that if steric occlusion of ribosomes by NusB/E binding is sufficient for repression of suhB, it would not require assembly of a complete Nus factor complex, since NusB/E alone has a high affinity for BoxA RNA\(^4\). Hence, we constructed suhB-lacZ translational fusions where the native promoter is replaced by a T7 promoter. Previous studies showed that gene regulation involving \(\lambda\) N or NusG is lost when \(E.\ coli\) RNAP is substituted with bacteriophage T7 RNAP\(^{20-22}\), suggesting that T7 RNAP does not interact with Nus factors; hence, transcription of this suhB-lacZ fusion by T7 RNAP would not be associated with formation of a complete Nus factor complex. We grew cells at 37 °C, 30 °C, or room temperature (23 °C), since the transcription elongation rate of T7 RNAP is similar to that of \(E.\ coli\) RNAP at room temperature, but considerably higher at 37 °C\(^{23,24}\). At all temperatures, we detected robust expression that was dependent upon expression of T7 RNAP in the same cells. However, we observed no effect on expression of mutating the boxA (Fig. S5). We conclude that efficient BoxA-dependent repression of suhB requires assembly of a complete Nus factor complex.

**Salmonella enterica suhB has a functional BoxA**

Phylogenetic analysis of the region upstream of the suhB gene indicates that the boxA sequence is widely conserved among members of the family Enterobacteriaceae (Fig. 2A; Fig. S2-3), suggesting that BoxA-mediated regulation of suhB occurs in these species. To investigate this possibility, we used ChIP of FLAG-tagged SuhB to measure association of SuhB with the suhB upstream region in \(S.\ enterica\) subspecies enterica serovar Typhimurium. We detected robust association of both RNAP (\(\beta\) subunit) and SuhB with the suhB upstream region (Fig. S6A-D), indicating that the suhB mRNA contains a functional BoxA. We also failed to
detect association with a previously reported cryptic BoxA within the hisG gene (Fig. S6), consistent with the sequence of this element differing at a critical position from the BoxA consensus (Fig. S2).

**BoxA-mediated regulation and Nus factor autoregulation are phylogenetically widespread phenomena**

Aside from their role in lambdoid phage, Nus factors have historically been considered dedicated regulators of rRNA expression. Our discovery of suhB as a novel regulatory target of Nus factors suggests that BoxA-mediated regulation may be more extensive. BoxA sequences in rRNA are known to be highly conserved.

Based on the boxA sequences from *E. coli* rRNA and suhB loci, and a previous analysis of sequences required for BoxA function in *E. coli*, we derived a consensus sequence (GYTCTTTAANA) that is likely to be applicable to almost all γ-proteobacteria. We searched for perfect matches to this sequence in 940 sequenced γ-proteobacterial genomes. We then selected sequence matches that are positioned within 50 bp of a downstream start codon for an annotated gene. Thus, we identified 407 putative BoxA sequences from 314 genomes, with between 0 and 7 instances per genome (Table S2). We determined whether any gene functions were identified from multiple genomes. To minimise biases from the uneven distribution of genome sequences across different genera, we analysed gene functions at the genus rather than species level. Across all the species analysed, we identified 36 different gene functions with at least one representative from one genus. Strikingly, we identified 34 of 55 genera in which at least one species has a putative boxA sequence within 50 bp of the start of an annotated suhB homologue. We identified three additional genera in which at least one species has a putative boxA within 50 bp of the start of an unannotated suhB homologue, and one genus with a species in which the suhB homologue has a putative boxA 82 bp from the gene start. Thus, our analysis reinforces the notion that BoxA-mediated regulation of suhB is highly conserved (Fig. 2A and S3). Three other gene functions were represented in multiple genera: prsA (encodes ribose-phosphate pyrophosphokinase) and rpsJ (encodes NusE) were each found in three genera, and genes encoding ParE-like toxins were found in two genera. We also identified two genera with species in which rpsJ is predicted to be a downstream gene in an operon where the first gene in the operon has a putative boxA <50 bp from the gene start.
**BoxA-mediated regulation of a toxin-antitoxin system in *Citrobacter koseri***

Bioinformatic analysis strongly suggested that BoxA-mediated regulation is evolutionarily widespread and extends to genes other than *suhB*. To determine whether Nus factors regulate genes other than rRNA and *suhB* in other species, we selected one putative BoxA-regulated gene identified by the bioinformatic search for *boxA*-like sequences: *CKO_00699* from *C. koseri* (Fig. S2). *CKO_00699* is predicted to encode a ParE-like toxin, part of a putative toxin-antitoxin pair. A putative *boxA* was observed upstream of a homologous gene in *Pasteurella multocida*, suggesting conserved BoxA-mediated regulation. We reasoned that if *CKO_00699* is a genuine target of Nus factors, it would likely retain this regulation in *E. coli*, since Nus factors are highly conserved between *C. koseri* and *E. coli* (e.g. the amino acid sequence of NusB is 97% identical and 100% similar between the two species). Hence, we constructed a transcriptional fusion of *CKO_00699* to *lacZ* and measured expression in *E. coli*. Note that we included a mutation in *CKO_00699* (R82A) to inactivate the predicted toxin activity to prevent growth inhibition. The *lacZ* fusion included a strong, constitutive promoter \(^2^5\), and the sequence from *C. koseri* began at the predicted transcription start site, based on manual analysis of likely promoter sequences (Fig. 6). We measured expression of fusions with wild-type and mutant *boxA* (C4A) sequences (Fig. S2), in wild-type and ΔnusB strains. Mutation of the putative *boxA*, or deletion of *nusB* resulted in a substantial increase in expression, whereas mutation of the *boxA* did not affect expression in the ΔnusB strain (Fig. 6). We conclude that *CKO_00699* is directly repressed by a BoxA and Nus factors.
DISCUSSION

A model for BoxA-mediated repression of suhB

We have shown that premature Rho-dependent termination within the suhB gene is controlled by a BoxA and Nus factors. This likely serves as a mechanism for autoregulation of Nus factors, since SuhB is a critical component of the Nus machinery. Premature Rho-dependent termination of mRNAs has been recently recognized to be a widespread regulatory mechanism. Most regulation of this type occurs by alteration of mRNA accessibility around Rut sites. In the case of suhB, Rho-dependent termination occurs as a result of translational repression.

A function for Nus factors in promoting Rho-dependent termination is particularly striking because of their long association with antitermination. The contrasting effects of Nus factors on Rho-dependent termination in different contexts, and their role in promoting ribosomal assembly, highlight the flexibility in the function of these proteins. Our data indicate that translational repression of suhB by Nus factors is not due to occlusion of the S-D. Previous studies of Nus factors suggest that they form a loop between the BoxA in the RNA and the elongating RNAP. We propose that this loop prevents the 30S ribosome from accessing the S-D. Alternatively, association of NusG with NusE in the context of the Nus factor complex may prevent translation by blocking association of NusG with ribosome-associated NusE (S10).

Autoregulation of SuhB is strikingly similar to autoregulation of λ N. λ nutL is positioned ~200 bp upstream of the N gene. Binding of Nus factors and N to NutL results in translational repression of N. The distance between NutL and the S-D sequence is such that a simple steric occlusion model is insufficient to explain translational repression by N and Nus factors; the RNA loop formed between NutL and the elongating RNAP provides a straightforward explanation of repression. Although the gap between NutL and the S-D sequence for the N gene is considerably longer than the longest distance we tested for suhB (Fig. 5), the intervening sequence is highly structured, which may impact the compactness of the loop.
Although we have shown previously that Nus factors are not required to prevent Rho-dependent termination at rRNA loci\textsuperscript{12}, Nus factors have been shown to prevent Rho-dependent termination in artificial reporter constructs\textsuperscript{10,11,30,31}. Our finding that Nus factors promote Rho-dependent termination in \textit{suhB} further indicates that context determines the precise function of Nus factors. Hence, it is likely that there are additional sequence elements in \textit{suhB} that promote Rho-dependent termination, or that there are additional sequence elements in the artificial reporter constructs that prevent Rho-dependent termination.

**BoxA-mediated regulation beyond rRNA**

Our data support a widespread regulatory role for Nus factors, implicating them in regulation in both a wide range of species, and of a diverse set of genes, although within any given species there are likely only a few regulatory targets. Strikingly, ~25% of the gene functions associated with an upstream \textit{boxA} are known to be directly connected to translation. This is consistent with the established connection between Nus factors and ribosomal assembly\textsuperscript{3}, and suggests that the impact of Nus factors on translation occurs by regulation of a variety of genes. Moreover, our data suggest that NusE is autoregulated in phylogenetically diverse species. Although we did not identify any genomes where genes encoding other Nus factors have putative upstream \textit{boxA} sequences, we did identify a putative \textit{boxA} sequence upstream of \textit{ribH} in six different species of \textit{Pseudomonas}. In all cases, \textit{nusB} is the gene immediately downstream of \textit{ribH}, suggesting that \textit{nusB} is autoregulated in pseudomonads. Overall, we identified no species with a putative \textit{boxA} upstream of more than one Nus factor-encoding gene, and only 11 genera had no putative \textit{boxA} associated with any Nus factor-encoding gene. However, for five of these latter genera we were unable to identify a \textit{boxA} sequence upstream of the rRNA genes, suggesting that the BoxA consensus is different to that in \textit{E. coli}. Thus, our data strongly suggest that Nus factor autoregulation occurs in ~90% of gamma-proteobacterial species, and that typically, just one Nus factor is autoregulated. The evidence for autoregulation of SuhB, NusE and NusB, suggests that the levels of these proteins contribute to feedback loops that control the primary function of Nus factors: promoting
ribosomal assembly. Our observation of BoxA-mediated regulation of a ParE-like toxin in *C. koseri*
demonstrates that Nus factors regulate genes other than their own. Indeed, our bioinformatic analysis suggests
that genes of many functions may be regulated by Nus factors, with 36 gene functions represented in at least
one genus. Our list is conservative because (i) it does not consider the possibility of regulation by BoxA
sequences located >50 nt upstream of the gene start, which we know is possible (Fig. 5), (ii) it does not consider
non-coding RNAs, (iii) the BoxA consensus may be different in some of the species analysed, and (iv) gene
starts predicted by bioinformatic annotation pipelines may be incorrect.

**Conclusions**

Our data indicate that regulation by Nus factors extends to many genes beyond rRNA, and that Nus factor
autoregulation is an evolutionarily widespread phenomenon. Moreover, we have shown that Nus factors can
provide contrasting forms of regulation, depending on the context of the target; despite their long-established
function in antitermination, Nus factors promote Rho-dependent termination within *suhB*. Key questions about
the function of Nus factors remain to be addressed. What is the molecular architecture of the Nus factor
machinery? What are the specific RNA sequences that determine whether Nus factors prevent Rho-dependent
termination? How do Nus factors modulate the function of elongating RNAP? Our identification of novel Nus
factor target genes with novel regulatory mechanisms provides an excellent opportunity to address these
questions.
MATERIALS AND METHODS

Strains and plasmids

All strains, plasmids and oligonucleotides used in this study are listed in Table S3 and Table S4. Mutations in \( rpsJ \) and \( rho \) were P1 transduced into MG1655 \( \Delta \)lacZ (AMD054) \(^{34}\) and MG1655suhB-FLAG\(_3\) (VS066) \(^{12}\). \( E. \ coli \) MG1655suhB(boxA(C4T/T6C)), MG1655suhB(boxA(C4T/T6C))-FLAG\(_3\), and \( S. \) Typhimurium \( hisG \Delta A+3::thyA, hisG \Delta A+100::thyA suhB-FLAG\(_3\) \) strains were constructed using FRUIT \(^{35}\).

Plasmids pGB1-pGB36, pGB67-68 were constructed by cloning the \( suhB \) gene and 200 bp of upstream sequence into the pAMD-BA-lacZ plasmid \(^{34}\), creating transcriptional or translational fusions to lacZ. Plasmids pGB192-pGB193 included 200 bp of upstream sequence and 57 nt of \( suhB \) coding sequence followed by a stop codon. Fusions carrying \( boxA \) mutations were made by amplifying a \( suhB \) fragment from GB023 (boxA(C4T/T6C)) or by site-directed mutagenesis (boxA(C4A)); \( suhB \) start codon mutations (ATG\( \rightarrow \)CAG) were made using site-directed mutagenesis. Insertions between the \( boxA \) and S-D sequences were generated by cloning fragments of random non-coding sequence (‘GAACCTCCCATCTGGTGCGAGATAGTATGAAC’), modified from \(^{36}\), for insertions of up to 32 bp; 40-100 bp insertions carried a non-coding sequence from the 16S RNA gene in the reverse orientation (region from +1281 to +1380). The 5’ end of the insert remained the same, and inserted sequence was extended towards the S-D sequence (see Fig. S7 for details). Plasmid pGB116 was made by cloning the T7 RNAP gene with a S-D sequence into pBAD18 \(^{37}\). Plasmids pGB83-95 carried the \( suhB \) gene and 36 nt of the 5’UTR with wt or mutant \( boxA \), and a 100 nt insertion between the BoxA and S-D elements, where indicated. \( suhB \) was under the control of pT7 promoter and was translationally fused to lacZ reporter on pAMD-BA-lacZ plasmid \(^{34}\). Plasmids pGB109-110 were made by cloning CKO_00699(R82A) gene with wt or mutant \( boxA \) (C4A) and a constitutive promoter \(^{25}\); the toxin gene was transcriptionally fused to lacZ reporter on pAMD-BA-lacZ plasmid.
Isolation and identification of trans- and cis-acting mutants

The trans-acting mutant genetic selection was performed using pAMD115 plasmid carrying a suhB-lacZ transcriptional fusion in MG1655 ΔlacZ. Bacterial cultures were grown at 37 °C in LB medium. 100 μL of an overnight culture was washed and plated on M9 + 0.2% lactose agar. Spontaneous survivors were first tested for increased plasmid copy number using qPCR, comparing the Ct values of plasmid and chromosomal amplicons. Strains with increased copy number were discarded. To eliminate plasmid mutants, plasmids were isolated and transformed into a clean MG1655 ΔlacZ background and plated on MacConkey agar indicator plates; mutants forming red colonies (upregulated suhB-lacZ) were discarded. Chromosomal mutations were identified either by whole-genome sequencing, as described previously 12, or by PCR amplification and sequencing of nusB, nusE and nusG. The cis-acting mutant genetic screen was performed by cloning a mutant suhB DNA library, generated by an error-prone DNA polymerase Taq (NEB) with oligonucleotides JW3605 and JW3606, into the pAMD-BA-lacZ vector, which was transformed into EPI300 background (lac-; Epicentre). The mutant library included the entire promoter, 5’ UTR and gene. We selected mutants that were visibly upregulated on MacConkey agar plates and sequenced the insert to identify mutations.

ChIP-qPCR

Bacteria were grown at 37 °C in LB medium until OD₆₀₀=0.5-0.6. ChIP-qPCR was performed as described previously ³⁴, using monoclonal mouse anti-RpoB (Neoclone #W0002) and M2 monoclonal anti-FLAG (Sigma) antibodies. Occupancy units were calculated as described previously ¹², normalizing to transcriptionally silent regions within the bglB or ynbB genes in E. coli, and the sbcC gene in S. Typhimurium.

β-galactosidase assays

Bacterial cultures were grown at 37 °C in LB medium to an OD₆₀₀ of 0.5-0.6. 100 μL of culture was used for β-galactosidase assays, as described previously ³⁴. LB medium was supplemented with 0.2% arabinose when
pBAD18 or its derivatives were used. β-galactosidase activity units were calculated as 1000 X 
(A_{420}/(A_{600})(time_{min})).

**Western Blotting**

Bacteria were grown at 37 °C in LB to an OD_{600} of 0.5-0.6. Cell pellets were boiled in gel loading dye, 
separated on gradient polyacrylamide gels (Bio-Rad), and transferred to a PVDF membrane (Thermo 
Scientific). The membrane was probed with control mouse monoclonal anti-RpoC (BioLegend) antibody at 
1:4000 dilution, or mouse monoclonal M2 anti-FLAG (Sigma) antibody at 1:10000 dilution. Goat anti-mouse 
horseradish peroxidase-conjugated antibody was used for secondary probing at 1:20000 dilution. Blots were 
developed with Clarity™ Western ECL Substrate (Bio-Rad).

**Sequence alignment of suhB upstream regions**

We extracted 100 bp of upstream sequence for suhB homologues in 19 species of the family 
*Enterobacteriaceae*, and aligned the sequences using MUSCLE 38 (Fig. S3). To determine the % match to *E. coli* at each position, we added 1 to the number of perfect matches (to account for the *E. coli* sequence), divided 
by 20 (to account for the 20 species in the alignment), and converted to a percentage.

**Identification of putative boxA sequences in γ-proteobacterial genomes**

We searched all sequenced γ-proteobacterial genomes for annotated protein-coding genes with the sequence 
GYTCTTTAANA within the 50 nt upstream of the annotated gene start. We compared gene functions using 
COG annotations 39.
ACKNOWLEDGEMENTS

We thank Dave Grainger, Don Court, Todd Gray and Keith Derbyshire for helpful discussions and comments on the manuscript. This work was supported by the NIH Director's New Innovator Award Program, 1DP2OD007188 (JTW).
AUTHOR CONTRIBUTIONS

J.T.W., G.B. and N.S. designed the study. J.T.W. and G.B. wrote the manuscript. G.B., N.S., C.B., R.J., R.F.,
M.P., A.M.S. and A.S. generated experimental data. P.L. performed bioinformatic analysis. All authors
contributed to data analysis and interpretation.
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FIGURE LEGENDS

Figure 1. Transcription termination within suhB is dependent on Rho and Nus factors. RNAP (β) enrichment at suhB 5’ and 3’ regions was measured using ChIP-qPCR in wild-type MG1655, boxA(C4T/T6C), ΔnusB, nusE(A12E) or rho(R66S) mutant strains. Values are normalised to signal at the 5’ end of suhB. x-axis labels indicate qPCR amplicon position relative to suhB. Error bars represent ±1 standard deviation from the mean (n=3). A schematic depicting suhB gene, the transcription start site (bent arrow) and boxA (grey rectangle) is shown below the graph. Horizontal black lines indicate the position of PCR amplicons.

Figure 2. A functional BoxA in the 5’ UTR of suhB. (A) Sequence conservation of the 100 bp upstream of suhB and its homologues across 20 Enterobacteriaceae species. The transcription start site is indicated by a bent arrow, and the BoxA and S-D sequences are indicated. (B) List of boxA mutations that are associated with increased suhB expression. All single nucleotide changes are indicated by an arrow. Single underline indicates a mutation that was isolated in the absence of mutations anywhere else in the cloned region; other mutants included additional mutations outside the boxA. Double underline indicates that the boxA mutation was isolated in two or more independent clones. Critical position “-4” is indicated (See Fig. S2). (C) SuhB association with the 5’ end of suhB in wild-type (“wt”) and boxA mutant (“boxA C4T/T6C”) strains. SuhB-FLAG occupancy was measured by ChIP-qPCR using α-FLAG antibody. Error bars represent ±1 standard deviation from the mean (n=3).

Figure 3. Nus factors repress translation of suhB, leading to Rho-dependent termination within the gene. β-galactosidase activity of (A) transcriptional and (B) translational fusions of suhB to lacZ in wild-type cells, ΔnusB, nusE(A12E), or rho(R66S) mutants. The suhB-lacZ fusion had either a wild-type (“wt”) or mutant boxA (“C4T/T6C”). Data are normalized to levels in wild type cells. Error bars represent ±1 standard deviation from the mean (n=3). Schematics of constructs used in these experiments are depicted above the graphs. (C) and (D)
Western blots showing SuhB-FLAG protein levels in wild-type cells, *nusE*(A12E), *rho*(R66S) (C), and *boxA*(C4T/T6C) mutants (D). SuhB-FLAG was probed with α-FLAG antibody; RNAP β’ was probed as a loading control. Representative blots from at least three independent experiments are shown.

**Figure 4. The suhB BoxA overlaps the first of multiple Rut elements.** β–galactosidase activity of short (A) and full-length (B) *suhB* transcriptional fusion to *lacZ*. Constructs included either a wild-type sequence (“wt”), *boxA* mutation (“C4T, T6C”) and/or *suhB* start codon mutation (“ATG→CAG”), as indicated on the x-axis. 200 nt of the 5’ UTR was included in all constructs. Error bars represent ±1 standard deviation from the mean (n=3). Schematics of the constructs used for these experiments are depicted below the graphs.

**Figure 5. The effect on suhB-lacZ transcription levels of altering the distance between boxA and the S-D sequence.** β–galactosidase activity of wild-type (“wt boxA”; dark grey bars) and *boxA* mutant (“C4A”; blue bars) transcriptional fusions of *suhB* to *lacZ*, with increasing lengths of non-coding DNA inserted between the *boxA* and S-D sequences. The length of inserted sequence (nt) is indicated on the x-axis. Constructs include 200 bp of upstream sequence and a full-length *suhB* fused to *lacZ* in the pAMD-BA-*lacZ* plasmid. Note that the sequence of inserted non-coding DNA differs for constructs with insertion sizes of ≤32 bp and ≥40 bp (see Methods and Fig. S7 for details).

**Figure 6. Identification of BoxA elements in other bacterial species.** β–galactosidase activity of wild-type (“wt boxA”) and *boxA* mutant (“C4A”) transcriptional fusions of *CKO_00699* (*R82A* mutant, to avoid potential toxicity to *E. coli* in the absence of the anti-toxin) to *lacZ* in *E. coli* wild-type (“wt”; dark grey bars) or *nusB* deletion (“ΔnusB”; red bars) strains. *CKO_00699-lacZ* expression was driven by a constitutive promoter. Error bars represent ±1 standard deviation from the mean (n=3).
**Figure S1. Transcription termination within suhB is dependent on Rho and Nus factors.** This is an extended version of Fig. 1. RNAP (β) enrichment at regions across suhB was measured using ChIP-qPCR in wt MG1655, boxA(C4T/T6C), ΔnusB, nusE(A12E) or rho(R66S) mutant strains. All values are normalised to the signal at the 5’ end of suhB gene. x-axis labels indicate qPCR amplicon position relative to the suhB ORF. Error bars represent ±1 standard deviation from the mean (n=3). A schematic depicting suhB gene, the transcription start site (bent arrow) and boxA (grey rectangle) is shown below the graph. The six horizontal black lines indicate the position of the PCR amplicons.

**Figure S2. A list of relevant boxA sequences from E. coli and related bacteria.** Nucleotide positions are numbered 1-11 above the sequences. BoxA from rRNA is considered a consensus. A critical nucleotide important for Nus factor association is “C” at position 4\textsuperscript{13,23}, and the mismatch in the S. enterica putative hisG BoxA sequence is underlined. suhB and CKO_00699 boxA mutations used in this study are in bold.

**Figure S3. MUSCLE (v3.8) Alignment (CLUSTAL Format) of 100 bp regions upstream of suhB homologues in Enterobacteriaceae species.** Species names are indicated to the left of the alignment. Asterisks indicate positions that are 100% conserved across the 20 species.

**Figure S4. The effect of 100 nt insertion between boxA and the S-D on suhB-lacZ expression levels when the native promoter is absent.** β–galactosidase assay of wild-type (“wt boxA”; dark grey bars) and boxA mutant (boxA(C4A); blue bars) suhB translational fusion to lacZ. The length of inserted sequence is indicated on the x-axis. The native suhB promoter was replaced by a T7 promoter (“pT7”; see schematic above the graph). Additionally, bacterial cells carried an empty pBAD18 vector (T7 RNAP was not supplied in this assay). Cells were grown in the presence of 0.2% arabinose. Error bars represent ±1 standard deviation from the mean (n=3). Note that the β-galactosidase activity from the translational fusion construct shown here is
substantially lower than the activity from the equivalent transcriptional fusion construct with a native suhB promoter (Fig. 5, far right). Moreover, β-galactosidase activity from a wild-type suhB-lacZ translational fusion construct is ~8-fold higher than the activity from the equivalent transcriptional fusion construct (607±8 and 78±3 β-galactosidase activity units, respectively). We conclude that the majority of β-galactosidase activity for the suhB-lacZ transcriptional fusion with a native promoter and a 100 nt insertion (Fig. 5, far right) is due to transcription from the native promoter.

Figure S5. suhB expression by T7 RNAP abolishes BoxA-mediated translational repression. β-galactosidase assay of wild-type (“wt”) and boxA mutant (“C4A”) suhB translational fusions to lacZ. The native suhB promoter was replaced by a T7 promoter (“pT7”; see schematic below the graph). Additionally, bacterial cells carried a plasmid with either an empty pBAD18 vector or pBAD18 expressing T7 RNAP (as indicated on the x-axis). β-galactosidase activity was measured for cells grown in the presence of 0.2% arabinose to induce T7 RNAP expression at 37 °C, 30 °C or 23 °C as indicated in the legend. Error bars represent ±1 standard deviation from the mean (n=3).

Figure S6. Evidence for and against functional BoxA elements upstream of suhB and inside hisG gene in S. Typhimurium, respectively. Previous studies reported a functional BoxA within the S. Typhimurium hisG mRNA. This putative BoxA was reported as being functional only when hisG translation was abolished by mutation of the gene. Hence, we interrupted hisG upstream of the putative boxA by inserting the thyA gene 3 bp or 100 bp downstream of the start codon. RNAP (β) (A and C) and SuhB-FLAG (B and D) association with thyA, hisG and suhB was measured using ChIP-qPCR in a derivative of S. Typhimurium strain 14028s in which thyA was deleted at its native locus, and hisG was disrupted by insertion of thyA, replacing the first 3 (A and B) or 100 (C and D) nucleotides of hisG. x-axis labels indicate the qPCR amplicon used, with numbers corresponding to the schematics above the graphs. Error bars represent ±1 standard deviation from the mean.
(n=3). In the schematic, the suhB boxA and the putative hisG boxA are indicated by grey rectangles. Numbers above the arrows represent nucleotide positions relative to the hisG gene start (without thyA insertion). Horizontal black lines indicate the positions of PCR amplicons.

Figure S7. suhB gene sequence used in lacZ fusion constructs. Relevant features used in this work are indicated: transcription start site (bent arrow), boxA sequence (single underline), S-D (double underline), start (“ATG”) and stop (“TAA”) codons (bold). The non-coding DNA sequence inserted between BoxA and S-D (Fig. 5) is shown in the box above, and the arrow points to the position of the insertion. Underlined nucleotides indicate the 3’ ends of various insertions and correspond to the insertion size labeled in Fig. 5. suhB sequence used in lacZ fusions in Fig. 3A-B included from position -200 to the end of the gene, as indicated by dashed lines. The short suhB-lacZ transcriptional fusion from Fig. 4B included suhB sequence up to position +57, and an in-frame stop codon immediately after the gene fragment.

Table S1. List of nusB, nusE and nusG mutants isolated in the genetic selection for factors that repress suhB.

Table S2. List of all genes in γ-proteobacteria with a putative boxA sequence ≤50 bp upstream.

Table S3. List of bacterial strains and plasmids used in this study.

Table S4. List of oligonucleotides used in this study.
Figure 1

The figure shows a bar chart illustrating the occupancy units of RNAP α-β across different regions of the suhB gene. The x-axis represents the genomic positions (-17 → +104 and +699 → +798), while the y-axis represents occupancy units normalized to the 5' end. Different conditions are color-coded: wt, boxA(C4T/T6C), ΔnusB, nusE*, and rho*. The chart highlights the variation in occupancy under these conditions.
Figure 3

A

suhB transcriptional fusion

B

suhB translational fusion

C

D

β-Galactosidase activity

α-FLAG (SuhB)

α-β’
Figure 6

Diagram showing the genetic construct with the gene CKO_00699 inserted before lacZ. Below the diagram, a bar graph comparing β-galactosidase activity between wild type (wt) and ΔnusB conditions for two variants: wt boxA and boxA(C4A).