RNA-binding specificity of E. coli NusA

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Received March 18, 2009; Revised May 12, 2009; Accepted May 13, 2009

ABSTRACT

The RNA sequences boxA, boxB and boxC constitute the nut regions of phage λ. They nucleate the formation of a termination-resistant RNA polymerase complex on the λ chromosome. The complex includes E. coli proteins NusA, NusB, NusG and NusE, and the λ N protein. A complex that includes the Nus proteins and other factors forms at the rrn leader. Whereas RNA-binding by NusB and NusE has been described in quantitative terms, the interaction of NusA with these RNA sequences is less defined. Isotropic as well as anisotropic fluorescence equilibrium titrations show that NusA binds only the nut spacer sequence between boxA and boxB. Thus, nutR boxA5-spacer, nutR boxA16-spacer and nutR boxA69-spacer retain NusA binding, whereas a spacer mutation eliminates complex formation. The affinity of NusA for nutL is 50% higher than for nutR. In contrast, rrn boxA, which includes an additional U residue, binds NusA in the absence of spacer. The Kd values obtained for rrn boxA and rrn boxA-spacer are 19-fold and 8-fold lower, respectively, than those for nutR boxA-spacer. These differences may explain why λ requires an additional protein, λ N, to suppress termination. Knowledge of the different affinities now describes the assembly of the anti-termination complex in quantitative terms.

INTRODUCTION

Gene expression in Escherichia coli and its phage can be controlled at the level of transcription termination. The best-studied examples of this mechanism are the ribosomal operons (rrn) and the bacteriophage λ (1–3). Transcription of the E. coli rrn operons is in part regulated by suppression of termination (anti-termination) (4). Anti-termination in rrn is mediated by an RNA recognition sequence (AT) located just distal to the promoters, close to the 5′ end of the pre-rRNA transcript (Figure 1A). A number of factors, including NusA, NusB, NusE (ribosomal protein S10) and NusG, modify RNA polymerase (RNAP) at AT. The modified RNAP is insensitive to termination by Rho-dependent terminators that occur throughout the long pre-rRNA transcript. AT includes a highly conserved sequence (boxA) that binds NusB, NusE and NusB–NusE complex (5,6). Distal to AT is an additional conserved sequence (boxC) that is less well characterized, but is a specific binding site for NusA in Mycobacterium tuberculosis rrn (7). Two short oligo ribonucleotides derived from the boxC stem–loop motif bind exclusively to the two KH domains of NusA in a completely extended conformation, and adenine-backbone interactions with the trinucleotide sequence AUA are particularly critical for this interaction (8).

Gene expression in lambdoid phages is also controlled by anti-termination. The Nus proteins form a complex with and modify RNAP at the λ nutL and nutR sequences. nutL and nutR consist of boxA, a spacer, a stem–loop element (boxB), and boxC (Figure 1B). The rrn boxA (5′-UGCUCUUUA-3′) and the λ boxA (5′-CGCUCUUAA-3′) differ; the CUUUA of rrn boxA is thought to enhance anti-termination efficiency (9). λ and other lambdoid phages express N, an RNA-binding protein of the arginine-rich motif (ARM) family, that binds boxB (10–13). N is required for anti-termination on the λ chromosome (14). In both the rrn and λ anti-termination systems, the modified RNAP retains the ability to transcribe through multiple terminators. However, rrn anti-termination is effective only at Rho-dependent terminators, whereas λ anti-termination complexes are highly resistant to both Rho-dependent and Rho-independent terminators (15).

The nut sequences and the Nus factors are also utilized by the phage HK022 Nun protein, an ARM protein related to N, to arrest transcription on the λ chromosome (11,16,17).

NusA is essential in wild-type E. coli (18,19) but not in E. coli deleted for cryptic prophage (20). In addition to promoting anti-termination, it enhances RNAP pausing (21,22) and termination (23,24). These reactions may be...
promoted by contacts between NusA and the 3′OH end of nascent RNA (25). NusA consists of five functional subdomains: an N-terminal domain that interacts with RNAP (26), three RNA-binding domains, S1, KH1 and KH2 (8,27,28) and two C-terminal acidic domains, AR1 and AR2, that interact with N and the α subunit of RNAP, respectively (Figure 1C) (17,29,30). AR2 masks one or more of the RNA-binding domains, thereby preventing NusA interaction with RNA (31). Structures of homologous NusA proteins from Thermotoga maritima (T. maritima) and from M. tuberculosis were determined in the absence and presence of RNA, respectively. Both structures show NusA to be highly elongated (8,27,28). Although knowledge of NusA has increased in recent years, several key questions are still open: Does E. coli NusA bind specifically or non-specifically to RNA? What rrn or nut sequences are critical for NusA binding? Are there structural differences between the NusA–rrn and NusA–nut RNA complexes?

**MATERIALS AND METHODS**

**Buffers and reagents**

All fluorescence titrations were performed in 50 mM potassium phosphate, 100 mM NaCl, 10 mM β-mercaptoethanol, pH 7.6, unless otherwise stated. Oligodeoxynucleotides as well as fluorescently-labeled oligoribonucleotides were obtained from biomers.net (Ulm, Germany; Table 1) and used according to the manufacturer’s instructions.

**Plasmid construct, expression and protein purification**

The DNA sequence of the NusA RNA-binding domains from amino acid 132 to 348 (NusA–SKK) was cloned via the BamHI and NdeI restriction sites into amino acid 132 to 348 (NusA–SKK) was cloned via plasmid vector pET11a (Novagen). The soluble recombinant NusA-SKK protein contained an N-terminal 5xHis tag. NusA-SKK was expressed and purified according to published procedures (31). Briefly, E. coli strain BL21 (DE3) (Novagen) harboring the recombinant plasmid was grown at 37°C in LB medium (Luria-Bertani) containing ampicillin (100 µg/ml) until OD600 = 0.5 and then induced with 0.1 mM isopropyl 1-thio-D-galactopyranoside (IPTG). Cells were harvested 4 h after induction, lysed and purified as described (31). Finally, the protein was dialyzed against buffer as used for fluorescence measurements. The dialyzed protein was concentrated with Vivaspin concentrators (Vivascience, MWCO 10 000 Da). The identity and structural integrity of purified protein was analyzed by 19% SDS–PAGE as well as by CD- and NMR spectroscopy.

**NMR spectroscopy**

NMR spectra were recorded on Bruker DRX 600 MHz spectrometers with triple-resonance probes equipped with pulsed field-gradient capabilities. The sample temperature was 298 K. 1D 1H spectra were collected with water suppression using a 1-1 spin-echo pulse sequence including gradients.

**Fluorescence equilibrium measurements**

We used various RNA sequences corresponding to λ nut to rrnG boxA sequence (rrn BoxA) of the E. coli genome (Table 1). Fluorescence equilibrium titrations were performed using an L-format Jobin-Yvon Horiba Fluoromax fluorimeter equipped with an automatic titration device (Hamilton). Extrinsic fluorescence measurements were performed using an L-format Jobin-Yvon Horiba Fluoromax fluorimeter equipped with an automatic titration device (Hamilton). Extrinsic fluorescence measurements with 3′ 6-carboxy-fluorescein (6-FAM)-labeled RNA were performed in fluorescence buffer as above in a total volume of 1 ml using a 10 × 4 mm quartz cuvette (Hellma GmbH, Mühlheim, Germany). The excitation wavelength was 492 nm, and the emission intensity was measured at 516 nm applying a 500 nm cutoff filter. For anisotropic measurements, slit widths were set at 4.5 nm and 3.5 nm for excitation and emission, respectively. All titration measurements were performed at 25°C with 50 nM of fluorescently-labeled RNA. Following sample equilibration, at least six data points with an integration time of 0.8 s were collected for each titration point in the case of anisotropic measurements.

**Data fitting**

Isotropic as well as anisotropic data were fitted to a two-state binding equation to determine the equilibrium dissociation constant ($K_d$) using standard software. The anisotropy was calculated from:

$$A = f_{\text{complex}} A_{\text{complex}} + f_{\text{RNA}} A_{\text{RNA}}$$

where $A$, $A_{\text{complex}}$ and $A_{\text{RNA}}$ are the anisotropy values and $f_{\text{complex}}$, $f_{\text{RNA}}$ are the fractional intensities. The change in fluorescence intensity has to be taken into account, so that the bound fraction is given by:

$$[\text{complex}] = \frac{A - A_{\text{RNA}}}{(A - A_{\text{RNA}}) + R(A_{\text{complex}} - A)}$$

with

$$[\text{complex}] = \frac{(K_d + [P]_0 + [RNA]_0)2[RNA]_0}{\sqrt{(K_d + [P]_0 + [RNA]_0)2[RNA]_0} - 4[P]_0[RNA]_0}$$

where $A$ is the anisotropy; $A_{\text{RNA}}$ is the initial free anisotropy, $A_{\text{complex}}$ is the anisotropy of the protein–RNA complex and $P_0$ and $RNA_0$ represent the total protein and RNA concentrations, respectively. $R$ is the ratio of intensities of the bound and free forms.

**RESULTS**

The E. coli NusA protein includes a C-terminal domain that masks the RNA-binding region (17,26,29,31). To determine the interaction of E. coli NusA with different RNA substrates, we used a NusA construct (NusA–SKK) lacking the two acidic-repeat C-terminal domains AR1 and AR2, as well as the N-terminal domain (Figure 1C). These regions are not directly involved in RNA binding. Thus, E. coli NusA416, deleted for AR2, forms complexes...
with the rrnG leader region as well as with the M. tuberculosis nut RNA. Electrophoretic mobility-shift assays (EMSAs) showed that the truncated E. coli NusA protein bound nut-like RNA species with high affinity, whereas the specificity was significantly lower than that of the M. tuberculosis NusA (7). This prompted us to investigate the affinity of different RNA species to E. coli NusA using fluorescence measurements. To avoid possible false negatives due to protein binding too distal to the fluorescence dye to alter fluorescence signal intensity, we used anisotropic fluorescence titrations instead of isotropic fluorescence measurements. Fluorescence anisotropy can detect molecular interactions even when an isotropic fluorescence signal change is weak or absent (32). Furthermore, changes of the fluorophore environment can be neglected with anisotropic measurements since the results are related to the rotational correlation time of a macromolecule with a rigidly attached fluorophore (33).

An extended rrn boxA sequence has the highest affinity to NusA–SKK

We first turned our attention to three different RNA species, the rrnG anti-terminator region, λnutL and λnutR, all of which interact with NusA and the other Nus factors (4). rrn carries a stem–loop structure (boxB), boxA and boxC sequences. The boxB and boxC sequences of rrn are not required for anti-termination (1).

The boxA sequence of rrn differs from that of λ at the initial base and by the insertion of an additional U residue at the penultimate site, converting the rrn boxA to a consensus site. Conversion of λ boxA to consensus enhances N activity (34). The spacer sequence of rrn differs from both λnutL and λnutR, but all three spacers carry a conserved sequence of AUU (Figure 1). Interestingly, we find that the rrn cac-boxA-spacer sequence, which includes a CAC sequence just upstream to boxA, binds with higher affinity to NusA–SKK (Kₐ = 14 μM; Figure 2; Table 1) than either the λnutR boxA-spacer (126 μM) or the λnutL boxA-spacer (71 μM; Figure 2; Table 1).

Role of boxA flanking sequences in binding of NusA–SKK

In these experiments, we tested boxA sequences with flanking regions (Table 1). In the case of rrn, these included sequences between boxB and boxA (in capital letter), as well as sequences between boxA and boxC (spacer, in italics). In the case of phage λnutL and λnutR, the spacer separates boxA from boxB. We proceeded to further define the NusA–SKK interaction regions at λnutR, λnutL and rrn.

In the case of the λnut sites, we find that the λnutL spacer binds to NusA–SKK (24 μM), whereas boxA alone shows no association with the protein (Figure 3A). Similarly, the nutR spacer binds NusA–SKK with an affinity nearly identical to that of nutR boxA-spacer (Kₐ value ~137 μM; Figure 3B), whereas NusA–SKK binding to boxA could not be detected.

To validate this result, we analyzed nutR boxA-spacer sequences with mutations in the boxA region (34,35). The boxA5 and boxA16 mutations decrease N activity,
binds NusA–SKK less efficiently than /C21 a failure to bind NusB (37). Why NusA–SKK binding. Their phenotype instead may reflect are conserved at both /C21 nutL-spacer these residues to G completely abolished NusA binding to interaction partners. Indeed, transversion of This conservation suggests that these bases are important /C21 boxA nucleotides are shown in bold. Mutated nucleotides are underlined. Flanking regions of rrn-boxA are in capital letters. The spacer is shown in italic.

whereas the boxA69 mutation has little effect on anti-termination (36). Fluorescence titrations of the three mutant RNAs indicate that only boxA69 significantly increased the Kd value (>200 μM) for NusA–SKK complex formation, whereas boxA5 and boxA16 exhibited Kd values similar to that of wild-type boxA (~120 μM; Figure 4A). These data demonstrate that boxA mutations that affect anti-termination have a very limited effect on NusA–SKK binding. Their phenotype instead may reflect a failure to bind NusB (37). Why boxA does not interact with NusA–SKK

In contrast to boxA and flanking sequences, titration of λnutR boxB with NusA-SKK, showed no, or only

Table 1. 3’6-carboxylfluorescein (6-Fam)-labeled RNA oligonucleotides used in this study

| Oligonucleotide          | Sequence                          | Kd for NusA-SKK (μM) |
|--------------------------|-----------------------------------|----------------------|
| nutR boxA-spacer         | 5’-gcucuuacacauu-3’               | 126 ± 4              |
| nutL boxA-spacer         | 5’-gcucuuacacauu-3’               | 71 ± 4               |
| rrn cac-boxA-spacer      | 5’-CACacacuuacacauu-3’            | 14 ± 0.2             |
| nutR boxA               | 5’-gcucuu-3’                      | n.d.                 |
| nutR spacer              | 5’-gcacauuca-3’                   | 137 ± 17             |
| nutL spacer              | 5’-aaaauuuu-3’                     | 24 ± 2.2             |
| nutR boxA5-spacer       | 5’-gcucuuacacauu-3’               | 124 ± 7              |
| nutR boxA16-spacer      | 5’-gcucuuacacauu-3’               | 106 ± 4              |
| nutR boxA69-spacer      | 5’-gcacacauuca-3’                 | n.d.                 |
| nutL boxA-spacer (mut)  | 5’-gcucuuacacauu-3’               | n.d.                 |
| rrn boxA                 | 5’-gcucuu-3’                      | 194 ± 38             |
| rrn-upstream-boxA’ (I)  | 5’-CACacac-3’                     | 26 ± 0.8             |
| rrn-upstream (II)        | 5’-GCUCGCAC-3’                    | 30 ± 1.9             |
| rrn spacer (III)         | 5’-aaaauuuu-3’                     | 71 ± 3.3             |
| nutR boxB               | 5’-gcacacauuca-3’                 | n.d.                 |

boxA nucleotides are shown in bold. Mutated nucleotides are underlined. Flanking regions of rrn-boxA are in capital letters. The spacer is shown in italic.

Figure 3. Fluorescence anisotropy measurements with separated RNAs regions. In each titration 50 nM of 6-FAM-labeled RNA was used. (A) 50 nM of 6-FAM-labeled λnutL boxA-spacer (squares), λnutL boxA (circles), λnutL boxA-spacer (triangles) were titrated with NusA-SKK. Kd values of 71 μM and 24 μM were determined for λnutL boxA-spacer, λnutL boxA, respectively (solid lines). No Kd value could be fitted to λnutL boxA (see Table 1). (B) 50 nM of 6-FAM-labeled λnutR boxA-spacer (circles), λnutR boxA (squares), λnutR boxA (triangles) were titrated with NusA-SKK. Kd values of 126 μM and 137 μM were determined for λnutR boxA-spacer, λnutR boxA, respectively (solid lines). No Kd value could be fitted to λnutR boxA (see Table 1). (C) 50 nM of 6-FAM-labeled rrn boxA alone (open triangle), rrn cac-boxA-spacer (open square), rrn spacer I (open circle), rrn spacer II (filled circle), rrn spacer III (filled triangle) were titrated with NusA-SKK. Kd values can be seen in Table 1. No Kd value could be fitted to rrn boxA (see Table 1).
very weak, nonspecific protein–RNA interactions (Figure 5). To confirm that \(\lambda nut\ boxB\) does not interact with NusA–SKK, even at higher concentrations, we analyzed a sample containing both species with 1D-NMR. In contrast to \(\lambda nut\ boxA\), \(\lambda nut\ boxB\) forms a stable stem–loop structure allowing the detection of the slowly exchanging imino protons in the double-stranded stem region. The 1D-NMR spectrum of NusA–SKK in the absence of RNA shows a well-dispersed amide proton signal region, indicating a stably folded, highly structured protein (Figure 5A). The \(\lambda nut\ boxB\) 1D-NMR spectrum reveals signals in the range of 12–14 p.p.m., corresponding to the imino protons of the stem region (Figure 5B). Interaction between the stem region of \(\lambda nut\ boxB\) and NusA–SKK, would affect these readily observable imino proton signals. The observable signals, however, of both protein and RNA, were unchanged when incubated together, clearly indicating that no complex forms between NusA–SKK and \(\lambda nut\ boxB\) even at NusA concentrations in the high micromolar range (Figure 5C). The observed signal increase is due to the lower concentration of \(\lambda nut\) RNA after addition of NusA–SKK.

CONCLUSIONS

Mutational studies indicated that NusA as well as boxA play an important role in anti-termination (38,39). boxA forms a complex with NusB/NusE (5,6,37) and it was suggested that NusA links \(\lambda nut\ boxA\) and \(\lambda nut\ boxB\) by binding to both (37,40). Oddly, however, and in contrast to boxA point mutations, anti-termination was still efficient, and NusB-independent, in a boxA deletion mutant (36,41). Additionally, deletion of the initial three bases (cac) of the \(\lambda nut\) spacer did not affect anti-termination, whereas deletion of the initial six bases (cacauu) led to complete loss of anti-termination activity. In agreement with the X-ray structure of \(M.\ tuberculosi\) NusA with RNA and deletion studies, our fluorescence analyses
revealed that NusA recognizes a spacer sequence that includes the critical bases AUU (8,36). NusA interaction with the rrn, nutL and nutR boxA-spacer motif was demonstrated by mutational studies and in vitro binding assays (1,37,39,42), and NusA was also suggested to recognize RNA outside the nut region (19,27,37,43). Complex formation between NusA and spacer might promote binding of NusE/NusB to the adjacent boxA sequence, and the notion that NusA binds to the nut spacer region is now strongly supported by the present fluorescence titration data.

Differences between the rrn anti-terminator regions and nut have already been described (5,37). Berg et al. (1) showed that rrn boxA-spacer plus seven upstream residues were sufficient to suppress termination at Rho-dependent terminators. We show here that the upstream CAC sequence as well as the downstream spacer, bind NusA–SKK. This redundancy may be related to the fact that nut-dependent anti-termination, which suppresses both Rho-dependent and Rho-independent terminators, requires λN and boxB, whereas rrn anti-termination requires neither (37,44). In addition to greater anti-termination efficiency, the requirement for λN and BoxB allows regulation of λ anti-termination. Thus, λ N levels are controlled at the levels of transcription, translation and protein stability (45,46).

Note that the NusE/NusB complex binds to boxA with affinities in the nanomolar range (5), whereas the Kd values for NusA–SKK are in the micromolar range. We suggest that tight RNA binding by NusA may not be required since it is already bound to RNA and thus in close vicinity to nascent RNA. The nutL spacer sequence differs from that of nutR spacer (Table 1), and this difference is thought to account in part for the enhanced efficiency of Nun-mediated termination at nutL relative to nutR (Washburn, R.S. and Gottesman,M.E., unpublished data), and nutL boxA-spacer binds with significantly higher affinity to NusA–SKK than does nutR boxA-spacer. Both spacer sequences contain U’s at residues 13 and 14, implying that these bases are important interaction partners. As shown above, replacement of the U’s with G’s completely abolished NusA–SKK binding to nutL-spacer. From this and other data, the following picture of the assembly of the anti-termination complex at the nut RNA has evolved (Figure 6): After RNAP has synthesized nut RNA, NusE and NusB bind to boxA, and NusA binds to spacer facilitated by NusA AR2 interaction with the C-terminal domain of the z subunit of RNAP. λN protein binds to AR1 of NusA as demonstrated for N(34-47) (17,30), forming a weak helix at the protein’s N-terminus (17). This weak helix facilitates recognition of boxB (17). NusA interaction with RNA is thus stabilized by the AR2:RNAP interaction as well as by the AR1:RNAP interaction, relieving the requirement for tight binding of NusA to nut.

**ACKNOWLEDGEMENTS**

P.R. would like to thank the Columbia University Microbiology Department for their patience during his sabbatical stay in New York.

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