The Structure of the Coliphage HK022 Nun Protein-λ-phage boxB RNA Complex

IMPLICATIONS FOR THE MECHANISM OF TRANSCRIPTION TERMINATION*  

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Cornelius Faber‡§, Manuela Schärpf‡§, Thomas Becker‡, Heinrich Sticht, and Paul Rösch†  
From the Lehrstuhl für Biopolymere, Universität Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany

Nun protein from coliphage HK022 binds to phage boxB RNA and functions, in contrast to phage λ N protein, as a transcriptional terminator. The basic Nun-(10–44) peptide contains the boxB RNA binding arginine rich motif, ARM. The peptide binds boxB RNA and competes with the phage λ ARM peptide N-(1–36) as indicated by nuclear magnetic resonance (NMR) spectroscopy titrations. In two-dimensional nuclear Overhauser enhancement spectroscopy experiments boxB RNA in complex with Nun-(20–44) exhibits the same pattern of resonances as it does in complex with N peptides containing the ARM, and we could show that Nun-(20–44) forms a bent α-helix upon binding to the boxB RNA. The structure of the boxB RNA-bound Nun-(20–44) was determined on the basis of 191 intra- and 30 intermolecular distance restraints. Ser-24 is anchored to the lower RNA stem, and stacking of Tyr-39 and A7 is clearly experimentally indicated. Arg-28 shows numerous contacts to the RNA stem. Leu-22, Ile-30, Trp-33, Ile-37, and Leu-41 form a hydrophobic surface, which could be a recognition site for additional host factors such as NusG. Such a hydrophobic surface area is not present in N-(1–36) bound to boxB RNA.

Bacteriophage λ N protein plays an essential role in transcriptional antitermination in the two-phase early operons that are critical for phage development. The inhibition of termination at intrinsic and p-dependent terminators by N protein depends on recognition of an RNA element called nut1 (N utilization) on the nascent phage transcript and on four Escherichia coli host factors (NusA, NusB, NusG, and ribosomal protein NusE). Together they form a ribonucleoprotein complex that converts the RNA polymerase into a termination-resistant form upon binding (1, 2). N protein binds specifically with high affinity to phage λ boxB RNA, a 15-mer RNA hairpin containing a purine-rich pentaloop (3, 4).

Nun protein of phage HK022 is a transcription termination factor that acts, in contrast to other termination factors, highly template- and site-specific. Nun terminates transcription uniquely on phage λ templates (5), competing with λ N protein for a common binding site, nut boxB RNA (6). Like N protein, Nun requires additional host factors (NusA, NusB, NusE, and NusG) for efficient termination, whereas the presence of NusA alone inhibits the termination activity of Nun (7). Recently it has been proposed that Nun arrests transcription by anchoring RNA polymerase to DNA (8). Both Nun and N proteins belong to the family of arginine-rich motif (ARM) binding proteins. The structures of phage λ N ARM peptide-boxB RNA complexes and of a phage P22 ARM N peptide-boxB RNA complex have been solved by NMR (9–12). For both these phage peptides, a very similar mode of binding has been observed, with the peptides bound in the major groove of boxB RNA, which adopts a typical hairpin conformation closed by an apical tetraloop.

MATERIALS AND METHODS

Sample Preparation—Unlabeled 15-nucleotide boxB RNA was synthesized in vitro transcription using T7 polymerase, a synthetic DNA template (GCCCTTTTTCCAGGGCTATAGTGAGTCGTATTA; MWG-BioTech, Ebersberg, Germany), and unlabeled nucleotide triphosphates. The RNA was purified as described previously (12). Freeze-dried boxB was resuspended in water, applied on a size exclusion column (NAP, Amersham Pharmacia Biotech) for desalting, and freeze-dried again. Nun-(10–44) and Nun-(20–44) were purchased from Biosyntan (Berlin, Germany). 15-N-labeled N-(1–36) peptide was expressed and purified as described previously (12). 1:1 complexes between either Nun-(10–44), Nun-(20–44), or N-(1–36) and boxB were generated by addition of small volumes of concentrated (2 mM) peptide to boxB RNA (~0.2 mM). To increase concentration for two-dimensional experiments, Nun-(10–44) boxB RNA complex was freeze-dried and resuspended in 280 μl of H2O or D2O, and Nun-(20–44) was concentrated by centrifuge centrifugation to a final volume of 270 μl. The samples with a concentration of 3 mM and 1.5 mM, respectively, were transferred into Shigemi microtubes.

NMR Spectroscopy—All NMR experiments were recorded at 25 °C for the Nun-(10–44) boxB RNA complex and at 30 °C for the Nun-(20–44) boxB RNA complex on a Bruker DRX 600 spectrometer equipped with 1H/13C/15N probes and triple-axis pulsed field gradient capabilities. For resonance assignment correlated spectroscopy (COSY), total coherence spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed using standard techniques for recording and water suppression (13). For the Nun-(10–44) boxB RNA complex, TOCSY experiments were recorded with 40 and 80 ms mixing time, and NOEY experiments were used for 80 and 200 ms mixing time, respectively. For the Nun-(20–44) boxB RNA complex, TOCSY experiments were recorded with a mixing time of 80 ms, and NOEY experiments were recorded with mixing time 150 and 300 ms. 1H N HSQC spectra were recorded with the fast HSQC pulse scheme (14). All NMR data were analyzed with the NDSee (SpinUp Inc., Dortmund, Germany) and XWINNMR (Bruker, Karlsruhe, Germany).
Structure of Nun-boxB RNA Complex

Program packages augmented with in-house-written routines. Proton chemical shifts were referenced to external 2,2-dimethyl-2-silapentane-sulfonic acid. The chemical shifts of the \(^{15}\)N resonances were referenced indirectly using the \(^{15}\)N/\(^{1}H\) \(\Xi\) ratio of 0.10132905 of the zero-point frequency at 298 K (15).

Interproton distance restraints were obtained from two-dimensional NOESY spectra of both Nun peptide-boxB RNA complexes. NOE intensities were estimated semi-quantitatively on the basis of cross-peak intensities from NOESY spectra collected with 80 ms mixing time. The categories "strong", "medium", and "weak" were converted into distance constraints with upper limit of 2.7, 3.5, and 5.0 Å for peptide intramolecular NOEs (16). For peptide-RNA intermolecular NOEs, these categories were converted in distance constraints with upper limit of 3.0, 4.0, and 5.0 Å, respectively. NOEs that were only visible in the NOESY spectra of Nun-(10-44)-boxB RNA complex with 200 ms mixing time were classified as "very weak" with an upper bound of 6.0 Å (10). To improve the convergence of the structure calculation, the lower bounds of all NOE restraints were set to 0 Å (17).

Molecular Dynamics Calculations—Experimental data clearly indicate that the boxB RNA in the Nun-(10-44) complex is virtually identical to the boxB RNA in the N-(1-36) complex. We thus used the structure of the boxB RNA in the N-(1-36)-boxB RNA complex that we determined earlier (12) (Protein Data Bank entry 1qfq) as a fixed template for all molecular dynamics calculations.

All structure calculations were performed using a modified ab initio simulated annealing protocol with an extended version of X-PLOR 3.851 (18). The calculation strategy, which was described in detail previously (19), included floating assignment of prochiral groups (20), a conformational data base potential term (21), and a reduced presentation for non-bonded interactions for part of the calculation (19).

The conformational search phase (60 ps of molecular dynamics at 2000 K) was followed by cooling from 2000 K to 1000 K within 40 ps, concomitantly increasing the force constants for the non-bonded interactions and the angle energy constant for the diastereospecifically unassigned groups to their final values. In the next stage of the calculation, the system was cooled from 1000 K to 100 K within 30 ps, applying the high force constants obtained at the end of the previous cooling stage. To detect the energy minimum, 1200 steps of energy minimization were performed, the final 1000 steps without conformational data base potential. In the final round, 100 structures were calculated, and the 20 structures that showed the lowest energy and the least number of violations of the experimental data were selected for further characterization.

For geometrical analysis and investigation of secondary structure and structural parameters the PROCHECK (22) and NUCPLOT (23) programs were used. Quick visualization and graphical presentation of the structures were performed with the programs RasMol V2.6 (24) and SYBYL 6.5 (Tripos Ass.) (25), respectively. The coordinates were deposited in the Protein Data Bank (entry 1HJI).

**Fig. 1.** a, alignment of the Nun-(10-44) peptide and phage λ N-(1-22) sequences. Numbering is for Nun. dots indicate conserved amino acids, asterisks identical amino acids. b, one-dimensional NMR spectra of free Nun-(10-44), free boxB RNA, and the Nun-(10-44)-boxB complex recorded in H\(_2\)O/D\(_2\)O (9:1), 40 mM NaCl, and 50 mM potassium phosphate, pH 6.4.

**Fig. 2.** Nun-(10-44)-N-(1-36) competition experiments. All spectra were recorded in H\(_2\)O/D\(_2\)O (9:1), 40 mM NaCl, and 50 mM potassium phosphate, pH 6.4. a, one-dimensional spectrum of the imino protons of boxB RNA in presence of Nun-(10-44) and N-(1-36). N-(1-36) was added to the Nun-(10-44)-boxB RNA complex, asterisks indicate resonances that were observed only after addition of N-(1-36). b, HSQC spectrum of N-(1-36) in complex with boxB RNA. c, HSQC spectrum of N-(1-36) in presence of boxB RNA and Nun-(10-44). Boxes indicate regions with typical resonances. In box 1 the same resonances as in b are observed but with lower intensity; in box 2 resonances typical for free random coil N36 are highlighted; and in box 3 new resonances neither observed with free N36 nor in the N36-boxB complex are highlighted.
RESULTS AND DISCUSSION

One-dimensional NMR Spectroscopy—We have monitored complex formation between the Nun-(10–44) peptide and the 15 nucleotide boxB RNA by one-dimensional NMR spectroscopy. Free boxB RNA shows three imino proton resonances for G12, G13, and G14. Upon addition of Nun-(10–44) changes in chemical shifts of these resonances were observed, and two additional resonances for the imino protons of U5 and G6 were detected (Fig. 1). The pattern of resonances is identical to that observed for boxB RNA in complex with N peptides (11, 12), suggesting that boxB RNA adopts virtually identical conformations in the presence of either peptide. Spectra of P22 boxB RNA in complex with a P22 N peptide also exhibit an identical pattern of imino proton resonances (10). Structure comparison shows that λ and P22 boxB RNA are very similar in complex with their respective N peptides, with one base looped out of the apical pentaloop, allowing formation of a typical GNRA tetraloop structure (10–12). The indole NH resonance of the only Trp residue of N-(1–36), Trp-18, is shifted upfield by more than 1.0 ppm (12) in presence of boxB RNA due to its stacking with the aromatic ring of A7, whereas the indole NH resonance of the single Trp residue of Nun-(10–44), Trp-33, is shifted upfield by only 0.2 ppm upon boxB RNA complex formation. The comparatively small change in chemical shift for Trp-33 in Nun-(10–44) may be attributed to conformational changes within the peptide upon RNA binding and already suggests that Trp-33 does not stack with A7 of boxB RNA. In the spectral range above 9 ppm another new signal is observed, originating from Ser-24 NH. For the N-(1–36)-boxB RNA and the P22 N-boxB RNA complex a corresponding signal is observed originating from Ala-3 (12) and Ala-2 (10), respectively. Nun-(10–44) and N-(1–36) Bind boxB RNA with Similar Affinities—To compare binding affinities of Nun-(10–44) and N-(1–36) to boxB RNA we prepared samples containing boxB RNA and equal amounts of Nun-(10–44) and N-(1–36). One-dimensional NMR spectra showed two sets of imino proton resonances with similar intensities, one corresponding to the Nun-(10–44)-bound boxB RNA and the other corresponding to the N-(1–36)-bound boxB RNA (Fig. 2a).

HSQC spectra of 15N-labeled N-(1–36) in complex with unlabeled boxB RNA shows a well resolved spectrum (Fig. 2b). After addition of an equimolar amount of unlabeled Nun-(10–44) to the N-(1–36)-boxB RNA complex several new resonances originating from unbound N-(1–36) appear in the spectrum (Fig. 2c), and the resonances of the boxB-bound N-(1–36) are reduced to less than 50% of their original intensities. In addition to those resonances from unbound, unstructured N-(1–36), additional resonances that may correspond to a partially structured, but unbound N-(1–36) could be observed. The indole imino proton of Trp-18, for example, shows three resonances: one resonance at 9.2 ppm as observed in the N-(1–36)-boxB RNA complex and two resonances around 10.2 ppm, one of which corresponds to the random coil peptide (Fig. 2c). As both bound and unbound N-(1–36) are observed regardless whether Nun-(10–44) is added to the N-(1–36)-boxB RNA complex or vice versa and as both complexes can be distinguished as N-(1–36) was 15N labeled, we can safely conclude that N-(1–36) and Nun-(10–44) bind to boxB RNA with approximately the same affinity and are able to compete with each other.

Two-dimensional NMR Spectroscopy—To obtain further information about the three-dimensional structure of the Nun-(10–44) complex, we recorded homonuclear two-dimensional COSY, TOCSY, and NOESY spectra of the Nun-(10–44)-boxB RNA complex and the Nun-(20–44)-boxB RNA complex, respectively. The spectra of both complexes for RNA and the...
peptide sequence from Leu-22 to Asn-43 were highly similar. Sequence-specific backbone assignments for Nun-(10–44)-boxB RNA complex as well as Nun-(20–44)-boxB RNA complex could be performed completely.

Assignment of RNA Protons and Verification of the RNA Fold—In two-dimensional spectra all H6/H8, H1', pyrimidine H5, and cytosine amino protons could be assigned. Pyrimidine H5 and H6 were identified in two-dimensional COSY-spectra, cytidine amino protons could be assigned by intraresidual NOEs to their own H5, and purine H8 and all H1' were assigned by comparison with spectra of boxB RNA in complex with λ N-(1–36). In this way, putative assignments were also possible for some isolated ribose resonances in the loop region, which show characteristic upfield (A7) or downfield (A9) shifts. Adenine H2 could be assigned for A7 and A9, and A11H2 could be identified by the strong NOE to U5H3 (Fig. 3).

NOEs indicating formation of Watson-Crick base pairs were observed for C2:G14, C3:G13, C4:G12, and U5:A11. Comparison of the Nun-(20–44)-boxB RNA and the λ N-(1–36)-boxB RNA NOESY spectra in the region of the imino protons show that the imino protons in Nun-(20–44)-boxB RNA are shifted downfield by about 0.05–0.2 ppm, whereas for all non-exchangeable protons no systematic change of the chemical shifts between the spectra could be observed (Fig. 3). Both spectra show an identical pattern of resonances suggesting virtually identical folds in the stem region.

The connectivities between the ribose and their own as well as their sequentially neighboring base were obtained from H1'–H6/H8, and H1'–H6/H8,–1–NOEs (26). As in the spectra of λ N-(1–36)-boxB RNA, no H1'–H6/H8,–1–NOE could be observed between A8 and A9. This and unusual downfield shifts of the resonances of A9 in the boxB-RNA complexes of both peptides suggest that A9 is looped out the Nun-(10–44)–boxB RNA complex. Spatial vicinity of A8 and A10 as well as A8 and A9 suggests that A9 is looped out of the Nun-(10–44)–boxB RNA complex. Spatial vicinity of A8 and A10 as well as A8 and A9 suggests that A9 is looped out of the Nun-(10–44)–boxB RNA complex.
is flexible and is not involved in RNA binding. Nun-(10–44) and Nun-(20–44) showed identical chemical shifts for the overlapping peptide. Thus, all further experiments were performed using Nun-(20–44).

For the Nun-(20–44) peptide-boxB RNA complex the sequence-specific resonance assignments could be performed by standard two-dimensional NMR methods (27). NOE cross-peaks in the backbone amide-amide region indicated the presence of helical structures for a large part of the RNA bound peptide. Unambiguous assignments of the amino acid side chain protons could be performed for all amino acids except Arg-25, Arg-27, Arg-29, Arg-32, Arg-36, and Lys-35 due to frequency degeneration and missing COSY-cross-peaks in the CαH region. Some arginine CαH could be assigned using the helix-typical $d_{ij}^H(i, i + 3)$ cross-peaks.

Nun-(20–44) Forms a Helix in the Complex—The analysis of the CαH chemical shift index (28) gave first evidence for a helical structure from Ser-24 to Asn-43. For this region, helix-typical ($i$, $i + 3$) and ($i$, $i + 4$) NOEs could be observed throughout the sequence (Fig. 4), in agreement with the observation of only very weak cross-peaks in the TOCSY-spectra for Leu-22 and subsequent residues, probably due to very small $3^J_{HH}$ coupling constants.

NOE Assignment and Structure Determination—To resolve ambiguities in NOE assignments, an iterative procedure for structure calculation was performed. Initially, only those NOEs that could be assigned unambiguously were used for molecular dynamics calculations. By verification of the resulting structures additional NOEs could be assigned, for example NOEs from the Trp-33 aromatic side chain to either Ile-30 or Ile-37.

Intermolecular NOEs—30 intermolecular NOEs could be identified unambiguously in the NOESY spectra. For the β-protons of Ser-24 the same NOEs with C2 and C3 are observed as...
than 0.13 Å. None of these structures showed NOE violations larger than 0.5 Å.

Aromatic protons of Tyr-39 show the same NOEs with A7 as seen in boxB (Fig. 5).

Various NOEs have been assigned between Trp33 aromatic protons and aliphatic protons of Ile-30 and Ile-37. In the D2O-NOESY spectra, an NOE between methyl protons of Leu-41 and Ile-37 could be observed. Additionally, the Leu-22 side chain is in spatial vicinity to Ile-30 and also contributes to the hydrophobic surface (Fig. 7b). All these residues are well defined in the structure calculations as shown in the overlay of 20 structures with the lowest overall energy (Fig. 7c). The network of hydrophobic residues forms a clear cut ridge (Fig. 7d) suggesting that the N-boxB- and the Nun-boxB-RNA complexes are recognized by different mechanisms by the target host cell factors.

It has been shown that NusA binds to the COOH-terminal region of Nun and that NusA alone without the other Nus factors inactivates Nun function as a terminator (7). NusA binding to boxB RNA can be nearly abolished by mutation of looped out nucleotide A9 (29). Together with N protein, NusA strongly enhances antitermination, which is also strongly reduced in vivo by mutations of A9 (30) implying that A9 plays a crucial role in antitermination. The current work thus may serve as a basis for the design of specific peptide and RNA mutants aimed at a better understanding of the crucial termi-
nation/antitermination regulation of the viral replication on a structural level.

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