NusA Is Required for Ribosomal Antitermination and for Modulation of the Transcription Elongation Rate of both Antiterminated RNA and mRNA*

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Ribosomal RNA (rRNA) is elongated twice as fast as mRNA in vivo due to the presence of antitermination sequences in the 5′ part of the rRNA transcripts. A number of Nus factors bind to RNA polymerase at the antitermination sites and help confer resistance to Rho-dependent termination of transcription. In this paper, the effects of the nusAc10 allele on the elongation rate of both mRNA and antiterminated RNA were investigated. The results indicate that NusA is required to achieve a high elongation rate of mRNA chains carrying the ribosomal antitermination boxA and that antitermination is defective when the rate of transcription elongation is decreased by the nusAc10 allele. Furthermore, the nusAc10 allele had no significant effects on the elongation rate of normal lacZ mRNA during steady state growth, but it abolished the inhibition of lacZ mRNA elongation by guanosine 3′,5′-bis(diphosphate) (ppGpp). These results suggest that NusA is the component of the transcription elongation complex required for inhibition of mRNA elongation by ppGpp.

Messengers RNA chains are elongated at a rate of 40–50 nucleotides (nt)/s during steady state growth, and the elongation rate is reduced to 20 nt/s in the presence of high intracellular concentrations of ppGpp (1, 2), which accumulate during the stringent response (3). Ribosomal RNA, on the other hand, is elongated at a rate of 80–90 nt/s, and the rRNA chain elongation rate appears to be unaffected by the stringent response (1, 4). The high rate of rRNA chain elongation depends on the presence of the antitermination sequences in the rRNA transcripts, and the incorporation of such an antitermination sequence, i.e. the boxA sequence, into lacZ resulted in an increase in the RNA chain elongation rate during steady state growth from 40–50 nt/s to 70–80 nt/s and made lacZ mRNA chain elongation insensitive to inhibition by ppGpp (4).

The ribosomal antitermination boxes were identified on the basis of their homology to the nut sites of bacteriophage λ (5, 6). The antiterminated transcription complex of phage λ consists of RNA polymerase in association with the λ-encoded N-protein and the host factors NusA, NusB, NusE (S10), and NusG (7), but the antiterminated transcription complex for ribosomal RNA is less well defined. It has been shown that the complex contains NusB and NusG and that it probably contains NusA as well (8, 9, 10). Furthermore, factors other than the known Nus factors are needed for proper ribosomal antitermination in vitro (9).

In addition to being a part of the antitermination complex, NusA becomes associated with the core polymerase shortly after release of the σ factor (11, 12), but the complex constantly dissociates and reforms during transcription elongation (13). NusA has also been reported to induce pauses during in vitro transcription, to reduce the rate of elongation, and to stimulate termination at intrinsic terminators (7, 13–15).

We have investigated the effect of NusA on transcription elongation in vivo by measuring the RNA elongation rate in a strain containing the nusAc10 allele. Our results suggest NusA is required both for ribosomal antitermination and for maintaining the high elongation rate of antiterminated RNA, since the nusAc10 mutant showed a considerable level of premature termination of boxA-lacZ mRNA chains and was unable to sustain a high elongation rate of RNA chains carrying the boxA sequence.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The strains and plasmids used are listed in Table I.

The MC4100 derivatives, S06100 and S06681, were constructed to have the nusAc10 allele (16) linked with the tetracycline resistance marker, wxz::Tn10 (17). The first strain was constructed by P1 transduction of the nusAc11 wxz::Tn10 strain YN2458 (17) as the donor and the nusA+ strain S03829 (18) as the recipient. Resistance to tetracycline (5 μg/ml) was selected at 32 °C. After testing for growth on LB broth at 43 °C, a temperature-resistant colony (S06100) was chosen. Strain S06681 was constructed by transduction of the nusAc10 strain, S06075 (14), with a P1 phage lysate grown on S06100 (wxz::Tn10), selecting for resistance to tetracycline on LB broth at 39 °C and isolating a colony unable to grow at 25 °C.

The isogenic pair of strains, S06718 and S06719, was constructed to have the nusAc10 mutation in the genetic background, MAS90, previously used for measurements of transcription elongation rates (1, 2, 4, 19). To facilitate this construction, the recombination-deficient strain, MAS78, was transformed with plasmid pMAS53, which encodes resistance to chloramphenicol, harbors a functional recA gene, and has a temperature-sensitive replication function (a generous gift from M. Sørensen, to be described elsewhere). The transformant, MAS78-pMAS53, was subsequently transduced with a P1 phage lysate grown on S06681 selecting for resistance to tetracycline (5 μg/ml) at 39 °C. The transductants were tested for the inability to grow at 25 °C (the nusAc10 phenotype), sensitivity to nitrofurantoin (the recA phenotype), and sensitivity to chloramphenicol (the loss of plasmid pMAS3). One cold-sensitive colony (a nusAc10 wxz::Tn10 mutant) and one colony able to grow at 25 °C (a nusA+ wxz::Tn10 strain) were chosen and subsequently mated with NF1830 for transfer of the F′ lacI32lacZ5 proAB5 episome by selecting for resistance to kanamycin on glucose minimal medium at 39 °C. These latter F′ derivatives were named S06718 and S06719.

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The abbreviations used are: nt, nucleotide(s); IPTG, isopropyl-1-thio-β-D-galactopyranoside; ppGpp, guanosine 3′,5′-bis(diphosphate); boxA, minimal sequence sufficient for ribosomal antitermination.

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Media and Growth—The bacteria were grown in shaking flasks in A + B medium (20) supplemented with 0.2% glucose, 1 μg/ml thiamine, and 100 μg/ml ampicillin at the indicated temperature. Isolecine starvation was induced by the addition of 0.4 mg/ml valine. Transcription from the PA1/04/03 promoter was induced by the addition of 1 mM IPTG (final concentration). Transformations with plasmids were performed by standard techniques. LB broth medium and solid media were prepared as described by Miller (21).

RNA Techniques—RNA sampling and purification was performed as described previously (2); dot blots and hybridization were performed as described by Vogel and Jensen (1), except that GeneScreen Plus hybridization transfer membranes from DuPont NEN were used for some experiments instead of GeneScreen membranes. The riboprobe plasmids employed and the procedure for in vitro transcription in the presence of [α-32P]UTP (DuPont NEN) have also been described (1, 2). The radioactivity on the dot blots was quantified on an Instant Imager (Canberra-Packard).

Determination of Transcription Elongation Rates—The transcription times of the lacZ gene on pUV12 and of the boxA-lacZ construct on pUV16 were determined as the time lag between induction of transcription initiation at the PA1/04/03 promoter by the addition of IPTG and detection of specific hybridization to a probe complementary to the 3′ end of the lacZ transcripts. Transcription elongation rates were calculated by dividing the length of the transcript (3117 nt for pUV12 and 3104 nt for pUV16) with the measured transcription time (2, 4). While the transcription times measured in these experiments were very reproducible, the absolute amount of radioactivity in the dots varied severalfold between identical experiments due to variations in the specific radioactivity of the probe, the number of times it was used, and the type of hybridization membrane employed, i.e., GeneScreen or GeneScreen Plus membranes. Therefore, the slopes of the induction curves are generally meaningless, and they can only be compared for experiments where hybridization was performed in parallel on the same membrane. In most cases, the transcription time was determined in two independent experiments, which were separated in time by days or weeks and included separate inoculation of cultures as well as growth of cells, sampling, preparation of RNA, and dot hybridization. However, to simplify the presentation, the two curves are shown in a normalized way where the radioactivity in the dots from one experiment was multiplied by a constant factor to superimpose the two induction curves on the other. The employed normalization factors ranged from 1 to 25 for the different panels in Fig. 2 and 3.

Determination of the Parameter r_s/r_t—Determination of the instantaneous rates of stable RNA initiations relative to total RNA (r_s/r_t) was performed as described by Baracchini et al. (22). Aliquots of the cultures (0.5 ml) were labeled with [3H]uridine (10 μCi, 43 Ci/mmol) for 60 s. Total RNA synthesis was determined as the amount of radioactivity in trichloroacetic acid-insoluble material, while ribosomal RNA synthesis was quantified by filter hybridization with plasmid pKK3535 DNA (23) or pBR322 for determination of background. [32P]-Labeled Escherichia coli RNA was used as an internal control of hybridization efficiency.

RNA Accumulation—The differential rate of accumulation of RNA was measured photometrically as trichloroacetic acid-precipitable, NaOH-labile material absorbing UV light at 260 nm as described by Vogel et al. (24).

Determination of Nucleotide Pools—This was performed using a medium containing 0.3 mM [32P]phosphate (~200 Ci/mmol) as described by Vogel et al. (24). The amount of radioactivity incorporated into the nucleotides was quantified on an Instant Imager (Canberra-Packard).

**RESULTS**

To test the effect of NusA on the rate of transcription elongation in vivo, we used the conditionally lethal nusAcs10 mutation, since the nusA gene can only be deleted in Rho-deficient strains (25). Strains harboring the nusAcs10 mutation are cold-sensitive and unable to grow at temperatures below 30 °C (16). The mutation was also shown to cause a defect in N-mediated transcriptional antitermination in bacteriophage λ (16), and it has been proposed to impose a defect in ribosomal antitermination, because it causes an increase in the rate of synthesis of tRNAs encoded outside the ribosomal operons, relative to those encoded inside these operons (10). The nusAcs10 mutant, SØ6718, and the isogenic nusA- strain, SØ6719, were grown in a glucose minimal medium at 34 °C, which is a semipermissive temperature for the nusAcs10 mutant. The generation times were 60 min for the wild type and 72 min for the nusAcs10 mutant.

Transcription elongation rates were measured using the plasmids pUV12 and pUV16 (Fig. 1), which both contain lacZ inserted behind the strong IPTG-inducible PA1/04/03 promoter. Plasmid pUV12 contains a normal lacZ gene, while pUV16 contains the minimal boxA-lacZ promoter, inserted in parallel on the same plasmid. Although boxA-lacZ has been previously shown to be both necessary and sufficient for proper antitermination in vitro and in vivo (6, 9), transcription rates were determined following induction of transcription initiation at the PA1/04/03 promoter by the addition of IPTG and hybridization to a probe complementary to the 3′ end of the lacZ transcripts (see “Experimental Procedures”).

Elongation of lacZ mRNA Chains in the nusAcs10 Mutant—The transcription time of the unmodified lacZ gene on pUV12 was 90 s during steady state growth of the wild type strain at
During the stringent response, provoked by inducing starvation for isoleucine, the transcription time increased to 160 s (Fig. 2C), in agreement with previous results (1, 2). For the \textit{nusAcs10} mutant the transcription time was 95 s (33 nt/s) during steady state growth (Fig. 2B), indicating that \textit{nusAcs10} does not disturb mRNA chain elongation during steady state growth. However, no inhibition of transcription elongation was observed during the stringent response, since the transcription time was 90 s under these growth conditions (Fig. 2D). These results suggested that the \textit{nusAcs10} allele abolished inhibition of transcription elongation by ppGpp, since the \textit{nusAcs10} mutant accumulated even more ppGpp than the wild type strain during the stringent response (Table II).

\textbf{TABLE II}

\begin{tabular}{|l|c|c|c|c|}
\hline
Nucleotide & \textit{nusA\textsuperscript{+}} & & & \textit{nusAcs10} \\
 & Steady state & Stringent response & Steady state & Stringent response \\
\hline
GTP & 460 & 330 & 640 & 300 \\
ATP & 950 & 970 & 995 & 1100 \\
CTP & 160 & 320 & 190 & 350 \\
UTP & 180 & 240 & 210 & 270 \\
ppGpp & 24 & 310 & 21 & 710 \\
\hline
\end{tabular}

Elongation of Antiterminated boxA-lacZ mRNA—For the \textit{nusA\textsuperscript{+}} strain, the transcription time of the antiterminated boxA-lacZ construct carried on pUV16 was 55 s both during steady state growth at 34 °C and during amino acid starvation (Fig. 3, A and C). This indicates that insertion of the antitermination boxA is sufficient both to elevate the elongation rate and render chain elongation insensitive to ppGpp, as previously observed (4). In the \textit{nusAcs10} mutant, however, the transcription time of the boxA-lacZ gene was 80 s during steady state growth (Fig. 3B), corresponding to an elongation rate of 38 nt/s, and it increased to about 95 s during the stringent response (Fig. 3D). The elongation rate observed during steady state growth for the \textit{nusAcs10} mutant was 38 nt/s, which is consistent with the previous observations (2).
state growth (38 nt/s) is similar to the elongation rate (33 nt/s) found for the normal lacZ mRNA and much lower than the elongation rate of the boxA-lacZ RNA observed in wild type cells (57 nt/s). These results suggest that the nusAcs10 mutant is unable to achieve a high rate of transcription elongation despite the presence of the boxA antitermination sequence.

Transcriptional Polarity—To determine the extent of premature transcription termination within boxA-lacZ during the stringent response, we quantified both the accumulation of 5' ends, which we interpret as the rate of initiation at the PA1/04/03 promoter, and the accumulation of 3' ends of the resulting RNA chain (Fig. 4). The slopes of these induction curves, which were made in parallel on the same membrane to ensure uniform hybridization, were identical during steady state growth and amino acid starvation, both for the wild type and for the nusAcs10 mutant (Fig. 4, A and B). This indicates that transcription initiation at the PA1/04/03 promoter is insensitive to ppGpp, as seen previously (4, 15).

For the wild type strain, the slopes of the curves representing the accumulation of 3' ends of the boxA-lacZ transcripts were also identical during steady state growth and amino acid starvation (Fig. 4C), but for the nusAcs10 mutant, the rate of accumulation of 3' ends of the boxA-lacZ transcript was reduced 4-fold after the onset of amino acid starvation (Fig. 4D). Therefore, we conclude that the minimal ribosomal boxA sequence is unable to suppress premature transcription termination in a strain harboring the nusAcs10 mutation.

The transcription elongation rates measured for the two transcripts under different growth conditions are summarized in Table III.

Control of RNA Synthesis during the Stringent Response—The inhibition of transcription elongation, normally observed during the stringent response, has been proposed by Jensen and Pedersen (26) to play a major role in the passive regulation of rRNA synthesis by sequestering RNA polymerase in the elongation phase and thus lowering the concentration of free RNA polymerase. Since the nusAcs10 mutant did not change the transcription elongation rate of mRNA, it could be used to test if the decreased mRNA elongation rate is important for the regulation of stable RNA synthesis during the stringent re-
The rate of total RNA synthesis, measured as the incorporation of \[^{3}H\]uridine into acid-insoluble material during 60-s pulses (Fig. 5A) or as the UV absorption of alkaline RNA hydrolysates (not shown), decreased strongly both in the \textit{nusA}^{+} strain and the \textit{nusAcs10} mutant after the onset of amino acid starvation. Furthermore, the parameter \(r_s/r_t\), which represents the fraction of de novo RNA synthesis devoted to stable RNA, decreased from 0.45 to 0.30 during the stringent response both for the wild type strain and for the \textit{nusAcs10} mutant (Fig. 5B).

This indicates that relative rates of initiation of mRNA and rRNA synthesis is normally regulated during the stringent response in the \textit{nusAcs10} mutant despite the constant RNA chain elongation rate.

\textbf{DISCUSSION}

Our results show that antiterminated boxA-lacZ RNA is elongated at a slower rate in the \textit{nusAcs10} mutant than in the wild type strain, and that the \textit{nusAcs10} allele abolishes the inhibition of normal lacZ mRNA chain elongation by ppGpp without affecting the elongation rate during steady state growth. The \textit{nusAcs10} allele contains two different mutations, which, in combination, confer cold sensitivity to the host (27). When the two mutations are separated, the \(\lambda\)-plating type is linked to the mutation at nucleotide 634 in the \textit{nusA} gene. It is therefore possible that the two different effects of \textit{nusAcs10}, i.e. the decreased elongation rate of antiterminated RNA and the resistance to ppGpp inhibition, are caused by different mutations.

The \textit{nusAcs10} allele prevented boxA-mediated suppression.
NusA-mediated Modulation of RNA Elongation

NusA protein to the transcription mixtures.

We found that the ratio between RNA and total RNA initiations (r/r t) decreased from 0.45 to 0.30 following induction of amino acid starvation, both in the nusAcs10 mutant and in the isogenic nusA + strain. This indicates that the ratio between RNA and mRNA synthesis is normally regulated in the nusAcs10 mutant during amino acid starvation and, thus, that the inhibition of transcription elongation and pausing by ppGpp does not play the major role in the regulation of RNA synthesis during the stringent response as proposed by Jensen and Pedersen (Ref. 26; but see Ref. 31). The nusAcs10 mutant, however, incorporated about 60% more [3 H]uracil in RNA during the pulse labeling under conditions of steady state growth (Fig. 5A). This may be the result of an increased labeling of the UTP pool resulting from the increased GTP pool in the nusAcs10 mutant, because uracil phosphoribosyltransferase is allosterically activated by GTP (32, 33). Alternatively, it may be that the nusAcs10 mutant has an increased capacity for total RNA synthesis despite the normal partitioning of RNA polymerase between mRNA and stable RNA initiations (r/r t). The elevated pool of ppGpp may be taken as an indication of an increased mRNA pool in the nusAcs10 mutant, which has previously been observed following induction of a stable version of the lacZ mRNA (34).

In summary, our results strongly indicate that NusA is part of the ribosomal antitermination complex, where it helps RNA polymerase to increase the elongation rate thereby preventing Rho-dependent premature termination, and they suggest a close relationship between a high elongation rate and the degree of antitermination. The results also indicate that NusA is part of the transcription elongation complex for mRNA synthesis and mediates the sensitivity of the elongating RNA polymerase to ppGpp.

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of polarity in the boxA-lacZ transcript, suggesting that the mutant is unable to antiterminate rRNA normally, in agreement with the observations of Sharrock et al. (10). Ribosomal antitermination is equivalent to the suppression of Rho-dependent transcription termination in the rrr operons (28, 29) and Rho-dependent termination has been proposed to depend on the kinetic coupling between the RNA polymerase and Rho (30). Thus, it is possible that the antiterminated RNA polymerase may simply escape Rho-dependent termination by running away from Rho as suggested by several groups (7, 28, 30). Our results are in agreement with this interpretation.

The observation that the nusAcs10 mutation caused resistance to inhibition of normal lacZ mRNA elongation by ppGpp indicates that NusA is part of the elongation complex for normal mRNA in vivo. This is in agreement with several previous observations from both in vivo and in vitro experiments (12–14). However, it also indicates that NusA is important for inhibition of mRNA chain elongation by ppGpp, although Kingston et al. (15) observed that ppGpp inhibited elongation of the early transcript of phage T7 in vitro without adding the
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