Mechanism of Transcription Activation at the comG Promoter by the Competence Transcription Factor ComK of Bacillus subtilis

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The development of genetic competence in Bacillus subtilis is regulated by a complex signal transduction cascade, which results in the synthesis of the competence transcription factor, encoded by comK. ComK is required for the transcription of the late competence genes that encode the DNA binding and uptake machinery and of genes required for homologous recombination. In vivo and in vitro experiments have shown that ComK is responsible for transcription activation at the comG promoter. In this study, we investigated the mechanism of this transcription activation. The intrinsic binding characteristics of RNA polymerase with and without ComK at the comG promoter were determined, demonstrating that ComK stabilizes the binding of RNA polymerase to the comG promoter. This stabilization probably occurs through interactions with the upstream DNA, since a deletion of the upstream DNA resulted in an almost complete abolishment of stabilization of RNA polymerase binding. Furthermore, a strong requirement for the presence of an extra AT box in addition to the common ComK-binding site was shown. In vitro transcription with B. subtilis RNA polymerase reconstituted with wild-type α-subunits and with C-terminal deletion mutants of the α-subunits was performed, demonstrating that these deletions do not abolish transcription activation by ComK. This indicates that ComK is not a type I activator. We also show that ComK is not required for open complex formation. A possible mechanism for transcription activation is proposed, implying that the major stimulatory effect of ComK is on binding of RNA polymerase.

Genetic or natural competence is a physiological differentiation state in which bacteria are able to take up exogenous DNA from the medium. This phenomenon has been best studied in the gram-positive soil bacterium Bacillus subtilis. Competence development is initiated at the onset of stationary growth as a result of a complex regulatory cascade. Through quorum sensing, environmental signals such as nutrient availability and cell density are sensed and interpreted (16, 34). Regulation by this cascade leads to the synthesis of the competence transcription factor (CTF), encoded by comK. Via an autoregulatory loop, ComK stimulates the expression of its own gene. In addition to this, ComK is required for the transcription of the late competence genes, comC, -E, -F, and -G, which encode the DNA binding and uptake machinery and addAB and recA, which are necessary for DNA recombination and integration (14, 31, 32, 37, 50, 69).

Previously, we have described the mechanism of binding of ComK to the promoter regions of specific competence genes (35, 69). A transcriptional fusion of the comG promoter with lacZ showed that in vivo expression of comG was completely abolished in a comK deletion mutant (68). In vitro transcription studies confirmed that ComK alone is capable of activating transcription at the comG promoter (35).

Transcriptional regulation by activators has been shown to affect transcription initiation at one or more of the following steps: (i) stimulation of RNA polymerase (RNAP) binding, (ii) stimulation of the isomerization to an open promoter complex, and (iii) helping in promoter clearance (1, 2, 44, 45). The free energy of reaction intermediates of transcription initiation can be limiting at any of these steps. Activator interactions could function in lowering the energy barrier of the rate-limiting step or steps in order to accelerate the overall transcription initiation reaction (27).

The possible interactions at the promoter site that could lead to transcriptional activation are generally divided into three separate levels: (i) direct protein-protein contacts between the activator protein and RNAP; (ii) conformational changes transmitted by the DNA upon binding of the activator; and (iii) additional contacts with other DNA segments than the core promoter sequence, such as interactions between the DNA sequence upstream of the activator binding site and the backbone of RNAP (15, 17). It has been postulated that the mechanism of activation depends on the architecture of the promoter as well as on the steps that are rate limiting in transcription initiation for that promoter (38). In the experiments reported here, we investigated the mechanism of ComK-dependent stimulation of transcription at the comG promoter. We demonstrate that RNAP binding to the promoter is stimulated by ComK and that stabilization of binding requires the presence of the upstream region of the promoter DNA. Furthermore, we show that C-terminal deletions in the α-subunit of RNAP do not abolish transcription activation by ComK. Isomerization to the open complex promoter is shown to be independent of ComK. The implications of these
results for the transcription activation mechanism of ComK at the comG promoter are discussed.

MATERIALS AND METHODS

Strains, plasmids, and plasmid construction. The plasmids and bacterial strains used in this study are listed in Table 1. Plasmid pAN-G-AT6 was constructed by long-range PCR using primers G+6F and G+6R with pAN-G as the DNA template. Ligation of the subsequent PCR product yielded pAN-G with a 6-bp insertion in between the ComK-binding sites as a template. The primers used in PCRs are listed in Table 2. Probes for use in electrophoretic mobility shift assays were made by PCR. A combination of the primers G1 and G2 was used to create a comG promoter fragment. A combination of the primers G2 and G1trn and primers G2 and AT-AT3 results in a truncated comG promoter fragment with two or three AT boxes, respectively. Probes with longer upstream DNA sequences were made with primers G7 and G2 for high-AT DNA of sipS or codY origin.

Purification of α-specific RNA polymerase and of α factor. To purify RNA polymerase, an overnight culture (5 μg of neomycin per ml) of B. subtilis NIG2001 (25) was diluted 100 times and grown at 37°C in 2 × tryptone-yeast medium and harvested at time 0. All subsequent procedures were performed at 4°C. Cells were collected by centrifugation for 10 min at 6,000 × g and washed with ice-cold buffer A (20 mM Tris-HCl [pH 8.0], 0.2 M NaCl, 1 mM 2-mercaptoethanol, 10% glycerol, 10 mM MgCl2). Cells were broken with a French press in buffer A containing 0.5 M phenylmethylsulfonyl fluoride (PMSF). Cell extracts were obtained by centrifugation for 20 min at 20,000 rpm in a SW50 rotor, after adding an additional 0.5 mM PMSF. The supernatant was diluted up to 10 times in buffer A and applied to a Talon resin column (Clontech) or Ni-nitrilotriacetic acid (Qiagen). The loaded column was washed with buffer A and buffer B (buffer A containing 5 mM imidazole) to remove nonspecifically bound proteins. Bound proteins were eluted by increasing the concentration of imidazole up to 50 mM in buffer A. Protein-containing fractions were diluted in low-salt buffer (20 mM

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RESULTS

ComK stimulates binding of RNAP at the comG promoter. The basal prokaryotic promoter consists of four critical elements: i.e., the −35 and −10 hexamers, the spacer length between these two hexamers, and upstream auxiliary elements. DNA sequences that resemble the consensus of such a core region are efficient binding sites for RNAP. Nevertheless, they may be poor promoters without the presence of activator proteins (7, 20, 24). The presence of a −35 consensus hexamer for RNAP is important for efficient binding of σ^A-RNAP to the promoter, since σ^A makes specific interactions with DNA at this region (30). In general, the homology score of promoter sequences correlates closely with the in vitro binding affinity of σ^A-RNAP (9, 20, 24). Since the comG promoter shows good homology to the σ^A consensus promoter (Fig. 1), the transcription properties of σ^A-RNAP at the comG promoter were analyzed by in vitro transcription studies (35). It was shown that in the absence of ComK, hardly any transcripts are formed, while in the presence of ComK, transcription is stimulated up to 50-fold, showing that ComK is sufficient and is required to activate transcription at the comG promoter.

Binding properties of σ^A-RNAP at the comG promoter were analyzed with electrophoretic mobility shift assays. RNAP was shown to bind to the comG promoter also in the absence of ComK. In the presence of ComK, the amount of complexes formed increased two- to fivefold (Fig. 2), resulting in a super-shifted complex. This result suggests that ComK stimulates binding of RNAP to the comG promoter.

RNA binding is stabilized by ComK through the upstream DNA region. When a truncated comG promoter fragment, lacking the DNA upstream of the ComK-binding sites, was used in an electrophoretic mobility shift assay under the same conditions, the stabilization of the RNA-polymerase-complex was abolished (Fig. 3A), although binding of ComK or RNAP alone was not disturbed. Therefore, it can be concluded that for stabilization of the complex, the DNA upstream of the ComK boxes is important.

Stimulatory effects of upstream DNA on transcription activation are known for several promoters (17, 61, 62). Often, a specific activation sequence, the UP element, can be distinguished, consisting of an AT-rich region located between −40 and −60 relative to the transcription start site (22, 23, 29). In the case of the comG promoter, this region is occupied by ComK binding, but the DNA-bending capacities of ComK suggest a possible specific sequence to be located further upstream of the promoter (59). To test whether the importance of the upstream DNA in the case of stable RNA binding at the comG promoter is a result of the presence of a specific sequence or of the structural presence of DNA, mutants were constructed in which the upstream DNA of comG was replaced by nonspecific DNA, with either a high GC content or a high AT content. Furthermore, two types of constructs were tested, which differed in the number of possible ComK-binding sites upstream of the promoter. Commonly, one K box, consisting of two AT boxes is present upstream of a ComK-activated gene (35). In the case of comG, an extra AT box is located one helical turn upstream of the common K box. In one set of mutants, called AT3, all three AT boxes were present, while in the other set of mutants, called AT2, only two boxes were present (Fig. 3C).

The different promoters were tested by electrophoretic mobility shift assays and in vitro transcription assays (Fig. 3B), showing that depletion of the third AT box resulted in an almost complete loss of stabilization of RNAP binding and transcription in the presence of ComK. This result indicates that the presence of the third AT box is of great importance for tran-
scription activation at the \textit{comG} promoter. However, the box alone is not sufficient to stabilize RNAP binding, since stabilization is still almost completely abolished when a truncated \textit{comG} promoter lacking the DNA upstream of the three \textit{AT} boxes is used. The replacement of the \textit{comG}-specific upstream DNA with either high-GC or high-AT DNA showed only a slight reduction of stabilization of RNAP binding, which never exceeded a 2- to 2.5-fold difference.

\textbf{ComK is not a type I transcriptional activator.} An important class of prokaryotic transcription factors mediates transcription activation through direct contacts with the RNAP. A preferred activation target is the C-terminal domain of the \( \alpha \)-subunit of RNAP (6, 21). In general, those activators binding at or upstream from position \(-60 \) relative to the transcription start site normally interact with the \( \alpha \)-subunit (41). To investigate whether ComK stimulates transcription through contacts with the \( \alpha \)-subunit, in vitro transcription assays were performed using RNAPs reconstituted with either wild-type \( \alpha \)-subunit or \( \alpha \)-subunits lacking the last 15, 37, or 59 amino acids from the carboxyl-end, respectively. Equivalent amounts of the reconstituted RNAPs were added to the transcription reaction mixtures, and the reaction products were separated by electrophoresis. The results demonstrated that the RNAPs containing deletion mutants of the \( \alpha \)-subunit were still stimulated by ComK (Fig. 4A), suggesting that direct protein-protein contacts between ComK and the \( \alpha \)-subunits are not re-
quired for transcription activation. However, the maximum level of transcription by RNAP reconstituted with the mutant /H9251-subunits was reduced approximately twofold compared to that of the wild-type polymerase (Fig. 4B). This suggests that the C-terminal domain of the /H9251-subunit is important for optimal transcription activity, as will be discussed.

Using electrophoretic mobility shift assays, it was also shown that ComK did not promote the binding of purified /H9251-subunits (wild type and deletion mutants) nor that of purified /H9268A to the promoter (results not shown). Thus, it can be concluded that direct protein-protein contacts between ComK and the /H9251- or /H9268-subunits of RNAP are not required for stabilizing RNAP binding to the promoter.

Open complex formation at the comG promoter is independent of ComK. The second step in transcription initiation is open complex formation. Competitor resistance is widely used as a functional assay for open complex formation (66, 71). Heparin challenge experiments indicated that the presence of both initiating nucleotides and independent of ComK.

ComK is not required for formation of open complexes at the comG promoter (Fig. 5A). Open complex formation was shown to be dependent on the presence of the initiating nucleotides. Only when ATP and GTP were added to the reaction mixtures did RNAP-promoter complexes become resistant to a heparin challenge. Upon addition of both nucleotides, an additional stabilizing effect on RNAP binding and an additional shift were observed, compared with the complexes formed in the absence or presence of only one of the nucleotides. This could be caused by the fact that in the presence of both initiating nucleotides a short abortive transcript can be formed (66). Likely, the formation of a short transcript stabilizes the binding of RNAP and causes a slightly altered migration pattern upon electrophoresis.

Normal open complex formation was also seen when the truncated comG promoter fragment with two AT boxes was used in the heparin challenge experiments (results not shown). These results confirm that when RNAP is bound to the promoter, open complex formation occurs upon the addition of nucleotides and independent of ComK.
Transcription activation is helix face dependent. To investigate whether the orientation of the ComK-binding sites is important for transcription activation, a mutant comG promoter fragment was created that contained a 6-bp insertion between the ComK box and the −35 hexamer. In this situation, the ComK-binding site is located on the opposite face of the DNA helix, and as a consequence, the bound ComK dimers are on the opposite face of the helix compared to the downstream RNAP. It has been found that ComK induces a bend in the DNA upon binding (35). In the comG+6 construct, this ComK-induced bend is present in the opposite direction compared to the wild-type situation.

The comG+6 promoter fragment was used in in vitro transcription assays, which showed abolishment of activation of transcription by ComK. In vivo, this promoter was placed in front of the lacZ gene in the amy locus of the B. subtilis chromosome. β-Galactosidase expression was abolished to the same level as in a comK deletion mutant (68; results not shown).

Electrophoretic mobility shift assays with comG+6 showed that stabilization of RNAP binding was disturbed, a situation comparable to that with the truncated comG promoters. Still, the initial level of RNAP binding in the absence of ComK was the same as that in the wild-type comG promoter, as was open complex formation (Fig. 5B). Binding of RNAP seemed to be lost when ComK bound to the other side of the helix. These results indicate a strict helix face dependency for transcription activation by ComK. It has been proposed that intrinsic or protein-induced DNA bends immediately upstream of a promoter site can activate transcription by looping the upstream DNA sequences around to interact with the backside of RNAP (56, 58, 59). This would explain both the requirement for the upstream region of the comG promoter and the helix face dependency.

DISCUSSION

Transcription initiation frequently requires the interaction of several DNA binding proteins that ultimately modulate the activity of RNAP. In competence development in B. subtilis, comK encodes the central regulator, also known as the CTF. ComK activates and binds specifically to the promoters of the late competence genes and the genes required for recombination. In vitro studies have shown that purified ComK alone is capable of activating transcription at the comG promoter. In this report, we describe the mechanism of this transcriptional activation.

In order to see in which step of transcription initiation ComK is involved, several approaches were taken. Using electrophoretic mobility shift assays, it was shown that RNAP can bind to the comG promoter also in the absence of ComK, but that the amount of closed complexes is stimulated up to fivefold when ComK is present (Fig. 2).

Stabilization of the RNAP-promoter complex in the presence of ComK was shown to be dependent on the DNA upstream of the ComK-binding sites. When this upstream DNA was deleted, binding of ComK and RNAP alone to the fragment was not disturbed (Fig. 3A), but the supershifted RNAP-promoter complex was no longer stabilized, suggesting that stabilization of RNAP binding is a result of bending of the upstream DNA by ComK, thereby enabling interactions between the DNA and RNAP.

Replacement of the comG-specific upstream DNA by either high-GC or high-AT DNA resulted in only a slight reduction in stabilization of RNAP binding and transcription (Fig. 3C). Several sequence comparisons were made between the upstream DNA of comG and those of other ComK-activated genes. No clear conserved sequences could be indicated, but a major difference between comG and other ComK-activated genes is the presence of an extra AT box upstream of comG. Binding assays and in vitro transcription studies comparing promoter fragments with either two or three AT boxes upstream of the comG gene showed the requirement of the third box for stabilization of RNAP binding and transcription in vitro (Fig. 3C). Previous footprinting studies by Hamoen et al. demonstrated that all three AT boxes are protected by ComK (35). The presence of this extra AT box might be the determinant that results in the large transcription at the comG promoter. Array studies indicated that comG transcription is the highest of all ComK-activated genes, and in vitro transcription studies with ComK-activated genes have thus far only been successful for comG (4, 33, 55). Studies with a truncated comG promoter that still contained all three AT boxes but lacked the upstream DNA no longer showed stabilization of RNAP binding, indicating that, in addition to the third AT box, the presence of more upstream DNA is required.

The requirement for upstream DNA correlates with the results shown in in vitro transcription assays with RNAPs reconstituted with the wild-type or C-terminal deletion mutants of the α-subunit. The results indicated that a direct interaction between the α-C-terminal domain (CTD) and ComK is not required for RNAP activation (Fig. 4), since ComK could still stimulate transcription by mutant RNAPs. Electrophoretic mobility shift assays showed that ComK is not able to recruit purified α-subunit or σ54 to the comG promoter, another indication that no significant contacts between ComK and RNAP are involved in activation.

Although transcription activation was not abolished in the reconstituted mutant RNAPs, a twofold reduction in maximal transcription was observed. Rowe-Magnus et al. (63) reported a similar observation for the transcription of the spoIIG promoter by Spo0A–P. They suggested an effect on the interaction of RNAP with promoter DNA by the α-subunit mutation. The CTD of the α-subunit is known to interact with additional promoter sequences (UP elements) to stabilize polymerase-DNA interactions at some promoters (17, 22, 23, 29, 61, 62). Although in the upstream region of comG, a clear UP element could not be demonstrated, it still is possible that specific AT-rich stretches in the upstream DNA interact with RNAP. In E. coli, the same residues of the α-CTD were found to be involved in interaction with activators such as CRP and promoter UP elements (52). If the α-subunit CTD would indeed help to stabilize the binding of RNAP to the promoter, it would explain why deletions in this domain disturb optimal transcription activity and why the presence of upstream DNA is important for optimal RNAP binding.

Although we cannot totally rule out the possibility that ComK interacts with RNAP through some other region of the enzyme than the α-subunits or σ54, like the β- or β′-subunit, we currently favor the notion that ComK activation of transcription
from the comG promoter is mediated via stabilization of RNAP binding through the upstream region of the promoter DNA.

Each step in the transcription initiation process is in principle a target for regulation by transcriptional activators (27). Activation can involve multiple interactions between a single activator molecule and the transcription machinery, each interaction being responsible for a specific mechanistic consequence. In fact, such multiple interactions have become a commonly observed feature in transcription activation (11, 38, 53). To advance our understanding of the effect of ComK on the transcription initiation process at the comG promoter, several experiments were performed to investigate in which step of transcription initiation ComK is involved.

When we inverted the orientation of the ComK- and RNAP-binding sites in the comG+6 promoter construct, stabilization of RNAP binding to the promoter was abolished. In the presence of ComK, no basal level of RNAP binding was observed, suggesting that binding of ComK to the opposite face of the helix hinders RNAP binding to the promoter. In addition, in vitro and in vivo transcription from this promoter was lost. Helix face dependency has been taken as evidence for cross-talk between RNAP and the activator protein (28). Since protein-protein interactions with the α- or σ-subunits of RNAP are not involved in transcription activation by ComK, it is likely that the orientation of the DNA bend caused by ComK binding is responsible for the helix face dependency. It has been proposed that activator-induced bending of the DNA upstream of the promoter facilitates caging of RNAP to optimize the promoter (2, 10, 59). We conclude that the mechanism of activation relies on contacts between the DNA upstream of the ComK-binding sites and the backside of RNAP. Similar findings have been reported for the gal and lac promoters (15) and for the CRP-dependent malt promoter (18).

For the in vitro transcription assays, supercoiled templates were used, because we found them to be approximately 20-fold more efficient in transcription than runoff transcription assays using the linear template (results not shown). The supercoiled state of the chromosome is known to affect the activity of many promoters (57). It is a fairly common phenomenon among prokaryotic promoters to be stimulated by DNA superhelicity (5, 60). The stimulatory effect of superhelicity of the template on transcription efficiency is also in agreement with our model. The influence of DNA bending on regulatory processes may be modulated by DNA superhelicity (26). Specifically, supercoiling and bending may synergistically enhance polymerase contacts by creating a defined DNA topology at the promoter site, a view also put forward by Zinkel and Crothers (72). Alternatively, DNA supercoiling may optimize the three-dimensional geometry of the DNA for correct alignment of the proteins and/or DNA sites, thus lowering energy barriers in transcription initiation (43).

The comG promoter has a strong resemblance to the B. subtilis consensus promoter sequence for σ70-dependent promoters (Fig. 1). In general, such consensus-like promoters stably bind RNAP and require alterations to accelerate the later steps of the transcription initiation pathway (19, 39). Therefore, ComK might also influence transcription initiation in one of the later stages after closed complex formation. The stabilization of the closed complex by ComK will, of course, contribute to accelerate the overall transcription process.

The second step in transcription initiation, open complex formation, was found to be independent of ComK. Addition of initiating nucleotides was sufficient to induce a heparin-resistant promoter complex. The formation of an open promoter complex is not disturbed when half a helical turn is inserted in between the promoter and the ComK boxes. All of this clearly indicates that open complex formation is not a rate-limiting step for transcription initiation at the comG promoter.

It has been suggested that consensus σ70 promoters that efficiently bind RNAP and that exhibit strong open complex formation may be limited in the subsequent movement of the polymerase to the elongating complex (12, 39). RNAP binding at these promoters generates a nucleoprotein complex that is too stable to allow promoter clearance (39, 51). Melting of the DNA strands in the promoter region in the presence of NTPs leads to an initiating complex that is trapped in short abortive transcript synthesis (48). The escape from this complex into an elongating transcription machinery involves major conformational changes, including loss of the promoter-specific contacts and the release of the σ factor (42, 49, 65). Escape from abortive initiation has been found to be rate limiting at several other prokaryotic promoters (48, 64). In the case of the comG promoter, initial experiments were performed to elucidate the role of ComK in promoter escape of RNAP. To distinguish between an effect of ComK on RNAP binding or promoter escape, ComK had to be added after the binding step. In this case, involvement of ComK in promoter clearance could not be demonstrated, since transcription levels were severely decreased when ComK was added in a later stage of the initiation process than in the binding step. Further research will be required to investigate whether bending of the upstream DNA
around ComK results not only in stabilization of RNAP binding, but also in creating optimal conditions for later steps in the transcription initiation process, like promoter escape.

The proposed model for the role in transcription activation by ComK is summarized in Fig. 6. Although RNAP is capable of binding to the comG promoter in the absence of ComK, binding is stimulated when ComK is present (step 1). In the case of the comG promoter, ComK can bind to three AT boxes, resulting in binding of the upstream DNA around ComK. This DNA probably interacts with the RNAP, thus stabilizing the RNAP-promoter complex (step 2). Further studies should be performed to see whether interactions between the upstream DNA and the backbone of RNAP also help to induce conformational changes in the promoter DNA and/or RNAP that are required for promoter clearance.

In this study, we have investigated the mechanism of transcription activation at the comG promoter, which differs from most other ComK-activated genes by containing a third AT box. However, we suggest that the transcription activation mechanism at promoters containing only two AT boxes is comparable to the model presented in this study. It is likely that the major effect of ComK is stabilization of RNAP binding via the upstream DNA region. We suggest that the function of the third AT box is mainly enhancing transcription levels at upstream DNA region. We suggest that the function of the most other ComK-activated genes by containing a third AT box is summarized in Fig. 6. Although RNAP is capable of binding to the comG promoter in the absence of ComK, binding is stimulated when ComK is present (step 1). In the case of the comG promoter, ComK can bind to three AT boxes, resulting in binding of the upstream DNA around ComK. This DNA probably interacts with the RNAP, thus stabilizing the RNAP-promoter complex (step 2). Further studies should be performed to see whether interactions between the upstream DNA and the backbone of RNAP also help to induce conformational changes in the promoter DNA and/or RNAP that are required for promoter clearance.

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References