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DNA strand separation during activation of a developmental promoter by the *Bacillus subtilis* response regulator Spo0A

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ABSTRACT **Spo0A is the central regulator of commitment to sporulation in *Bacillus subtilis*. Spo0A is a member of the response regulator family of proteins and both represses and stimulates transcription from promoters when activated. *In vivo* Spo0A activation takes place by phosphorylation and *in vitro* activation can be accomplished by phosphorylation or removal of the N-terminal domain of the protein. We have examined the mechanism of Spo0A stimulation of transcription from the promoter of the *spoIIG* operon. This operon encodes one of the first compartment specific sigma factors whose appearance regulates sporulation development. When activated Spo0A was incubated with RNA polymerase and a DNA fragment containing the *spoIIG* promoter, bases between −13 and −3, relative to the start site of transcription, were denatured. Addition of activated Spo0A or RNA polymerase alone did not induce denaturation. Heteroduplex templates that contained the nontemplate sequence of the wild-type promoter on both strands between positions −3 and −13 were efficiently transcribed without activated Spo0A. These data suggest that DNA strand separation is a two-step process and that the activation of Spo0A creates a form that interacts with the polymerase to induce the first of the two steps.**

When cultures of *Bacillus subtilis* reach high density and nutrient limitation they initiate the process of endospore formation. To create the endospore, the cell alters the process of cell division so that two cellular compartments (the mother cell and the forespore) are formed within one cell wall (reviewed in refs. 1–3). Each compartment follows a path of gene expression determined by the synthesis and activation of compartment specific sigma factors that associate with core RNA polymerase (2–6). The central regulator in the commitment to sporulation and the essential activator of the operons responsible for synthesis of the sporulation specific sigma factors σ^E (the *spoIIG* operon in the mother cell) and σ^F (the *spoIIA* operon in the forespore) is the product of the *spo0A* gene, Spo0A (3, 4, 7, 8) .

Spo0A is a member of the response regulator family of proteins (9–12). Response regulators are activated by phosphorylation by a cognate kinase in response to an input signal and thus the two proteins form a signal transduction pathway (13–16). Like most response regulators, Spo0A consists of two domains; an output domain that is the transcription activator and a receiver domain with amino acid similarity to the *Escherichia coli* protein CheY (7, 12–15). The receiver domain inhibits the activity of the output domain unless it is phosphorylated. Phosphorylation of Spo0A takes place through an elaborate phosphotransfer mechanism involving input from multiple kinases and two other intermediates, Spo0F and Spo0B (7, 8, 17). At least part of this phosphotransfer mechanism provides targets for specific protein phosphatases that can regulate the flow of phosphate to Spo0A to allow response to a variety of environmental signals (18–20)

Spo0A binds to DNA sequences (0A boxes) in the promoter regions of the operons that it activates (21, 22), and stimulates the rate of initiation of RNA synthesis from the target promoters (23–27). We have specifically investigated activation of the promoter for the *spoIIG* operon in the work reported here. This promoter is transcribed by RNA polymerase containing the major vegetative sigma factor, σ^A (28–30). The promoter contains the consensus −10 and −35 DNA binding sequences for σ^A, but the distance between the sites is 22 bp instead of the optimum 17 bp (30). The promoter also contains two sets of tandem 0A boxes. One set, located ≈100 bp upstream from the transcription start site, appears to be unnecessary for normal regulation *in vivo* (22). The other set, located between −53 and −37, overlaps the −35 consensus sequence and mutations in this site can increase or decrease promoter activity (22) .

Little is currently known of the mechanisms by which activated response regulators stimulate transcription. The activation mechanism for one response regulator, NtrC, has been examined in detail (31, 32) but that regulator is distinct because it controls the activity of a unique class of sigma factors, σ^{54} (33), and it is unclear what components of the mechanism will be general. We have used a combination of DNA structure probes and transcription from DNA templates containing artificially denatured regions to examine the mechanism of transcription activation by activated Spo0A. The results demonstrate that activated Spo0A cooperates with RNA polymerase to induce a specific denaturation event at the *spoIIG* promoter. This denaturation appears to be the rate limiting step in the initiation of transcription from the *spoIIG* promoter.

EXPERIMENTAL PROCEDURES

Mutagenesis of the *spoIIG* Promoter. The parent plasmid, pUCIIGtrpA (26) contains a 240-bp *Hin*dIII to *Bam*HI DNA fragment bearing the *spoIIG* promoter and 100-bp 5′ to the transcription start site, in which both the 0A binding sites reside. Two 35-mer oligonucleotide primers, MB1 (5′-CAGAGCTTGCTTATATGAATACTTGCAAGAAGGGG-3′) and MB2 (5′-CAGAGCTTGCTTATATGAATTGAAGCAAGAAGGGG-3′), were purchased from the Nucleic Acid and Protein Service Unit (University of British Columbia). These primers were complementary to the template strand from −26 to +9 with respect to the transcription start site and included the *Alu*I site (underlined). Each primer contained a stretch of 8 or 12 nucleotides identical, rather than complementary, to the template strand from −7 to −14 or −3 to −14. PCR products were generated using *Taq* polymerase (Boehringer Mannheim), pUCIIGtrpA as a template and a downstream primer (IIG2: 5′-GGGGATCCTCTAGAGTCA-

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3′), which anneals to the coding strand adjacent to the *Bam*HI site (Fig. 1).

The PCR products were cloned into the pGEM-T vector (Promega) to create pDR8 and pDR12. Inserts were verified by the dideoxy chain termination method by using the Sequenase 2.0 DNA sequencing system (United States Biochemicals). The *Hin*dIII–*Alu*I fragment that contains the two 0A binding sites, was isolated from pUCIIGtrpA and ligated to the *Alu*I–*Bam*HI fragment isolated from pDR8 or pDR12 to reconstitute the full-length promoter. The ligation mixture was used as template for a PCR reaction by using the downstream primer described above and an upstream primer complementary to the template strand, IIGA (5′-CCAAGCTTATCGACAAATTAA-3′), which annealed adjacent to the *Hin*dIII site. The resulting PCR product was cloned into pGEM-T to create pDR8IIG and pDR12IIG. Inserts were verified as described above. The *E. coli* strain DH5α [*hsdR17*($r_K^-m_K^+$) *supE44 thi-1 rexA1 gyrA* (Nal^r) *relA1* Δ*(lacZYA-argF)U169 (φ80lacZΔM15)*] was used for all of the necessary transformations. Plasmid preparations were done as described by Sambrook *et al.* (34) .

Construction of Heteroduplex Templates. The plasmids pUCIIGtrpA, pDR8IIG, and pDR12IIG were digested with *Hin*dIII and *Bam*HI, the 235-bp promoter bearing fragments were isolated by electroelution from agarose gel slices and ligated into the pBluescript SK^+ and SK^- vectors (Stratagene) digested with the same enzymes to produce pSK8IIG+, pSK8IIG−, pSK12IIG+, pSK12IIG−, pSKWTIIG+, and pSKWTIIG−. Single-stranded DNAs were generated by infection of *E. coli* JM101 cells [F′ *trad36 proA*$^+$ *proB*$^+$ *lacIq lacZΔM15/supE thi* Δ*(lac-proAB)*] containing pBluescript plasmids bearing the desired inserts with M13K07 helper phage. The DNA was collected by PEG precipitation, extracted extensively with phenol/chloroform, treated with RNase A and precipitated with ethanol (34). Complementary single-strands (10 μg each) were mixed and adjusted to 0.2 M NaOH in a final volume of 10 μl. Samples were heated at 37°C for 15 min, neutralized with HCl to 0.2 M, brought to a final volume of 27 μl in 1× *Pvu*II reaction buffer and incubated a further 15 min. *Pvu*II (30 units, GIBCO/BRL) was added and digestion allowed to proceed for 2 hr at 37°C. The promoter-bearing *Pvu*II fragments (675 bp) were purified on an agarose gel, electroeluted, and ethanol precipitated. The templates (MB8T, MB8NT, MB12T, and MB12NT) are shown in Fig. 2.

***In Vitro* Transcription Assays.** *B. subtilis* RNA polymerase was isolated as described by Dobinson and Spiegelman (35). Typically, preparations were 30–50% active under DNA saturating conditions. Spo0A, the proteins required to phosphorylate it, and Spo0ABD were a gift of J. A. Hoch (Scripps Research Institute, La Jolla, CA). The phosphorylation of Spo0A was carried out as described (26) .

Standard transcription reactions (20 μl final volume) contained as final concentrations: 4.0 nM template; 80 mM potassium acetate; 0.4 mM each of ATP, UTP, and CTP (fast protein liquid chromatography grade, Pharmacia); 0.005 mM

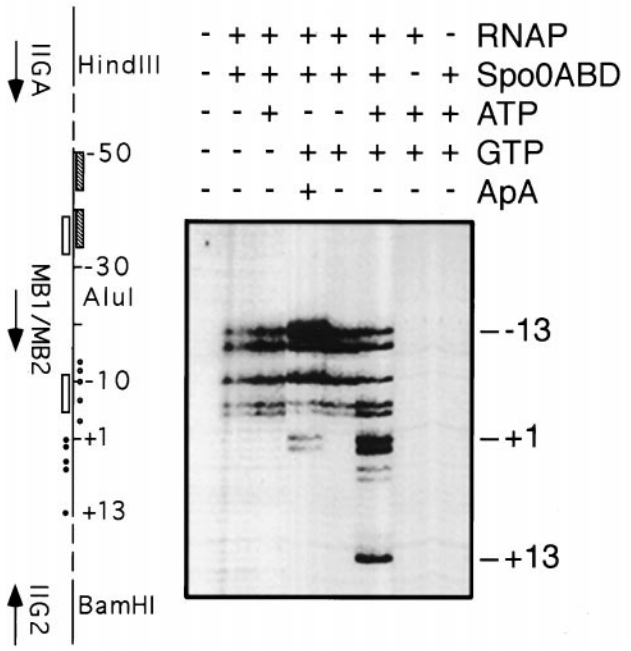

FIG. 1. Spo0ABD induces DNA strand separation at the *spoIIG* promoter. Transcription reactions containing 4 nM template DNA, labeled at the *Bam*HI site with [γ-^{32}P]ATP, 400 nM Spo0ABD, or 40 nM RNA polymerase (RNAP) or both in combination with different initiating nucleotides (as indicated above each lane) were treated with $KMnO_4$. Modified DNA was cleaved with piperidine, the cleavage products were analyzed on an 8% polyacrylamide gel containing 7 M urea and the gel was exposed to x-ray film. A representation of the *spoIIG* promoter in pUCIIGtrpA is shown on the left. Positions of restriction endonuclease sites and primers used in mutagenesis are indicated. The *Hin*dIII and *Bam*HI sites are not located to scale. Positions of 0A boxes (▨) and −10 and −35 sequences (□) are shown. Thymines sensitive to KMnO4 modification are indicated by dots to the right (sensitive in the absence of ATP plus GTP) and dots to the left (additional sites sensitive in the presence of ATP and GTP). Nucleotide positions are labeled relative to the start site of transcription (+1).

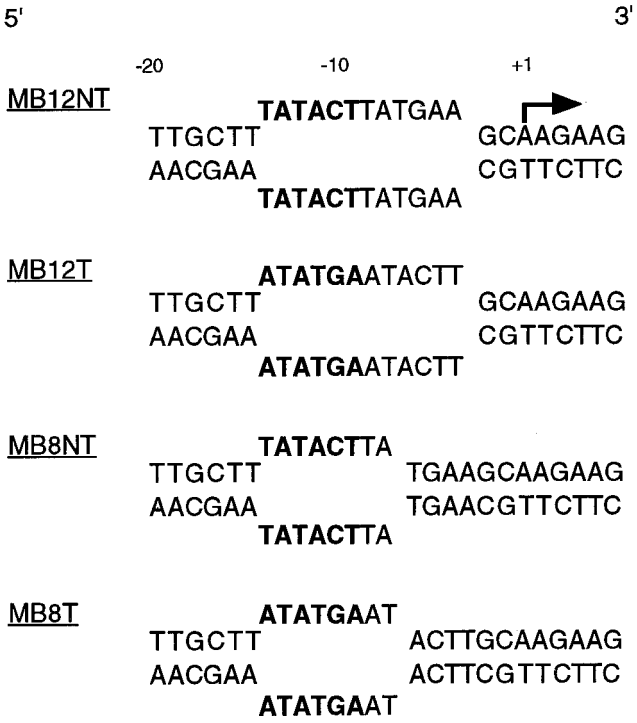

FIG. 2. Structure of the heteroduplex templates. Oligonucleotide primers containing either 8 or 12 nucleotides of template sequence corresponding to the −10 region of the promoter (−7 to −14 or −3 to −14, respectively) were used to mutate the *spoIIG* promoter sequence as described in *Experimental Procedures*. Single-stranded DNA of mutant and wild-type promoters were produced by infection of the *E. coli* clones with helper phage and annealed with the complementary wild-type or mutant strands to produce double-stranded DNA containing the desired region of heteroduplex. The noncomplementary regions of heteroduplex templates used in the subsequent transcription assays are shown. Mismatch bubbles (MB) of 8 or 12 bp were created containing the nontemplate sequence (NT) or the template sequence (T) of the −10 site (boldface) within the noncomplementary region. Not shown is the wild-type template produced by annealing the wild-type (+) and (−) DNA strands.

[α-^{32}P]GTP (20 Ci/mmol, 1 Ci = 37 GBq; New England Nuclear) in 1× transcription buffer (27). A 2-μl aliquot of an appropriate dilution of either Spo0A, Spo0A~P, or Spo0ABD was added to reactions containing template DNA, and the indicated initiating nucleotides in a 14-μl volume. After a 2 min incubation at 37°C, 2 μl of RNA polymerase was added, and after a further 2 min, a single round of RNA elongation was permitted by the addition of 2 μl of elongation mix (UTP, CTP, and 10 μg/ml, final concentration of heparin). After 5 min, reactions were stopped by the addition of one-fifth the volume loading buffer and transcripts were separated from unincorporated nucleotides by electrophoresis under denaturing conditions (27). Transcripts were localized by autoradiography (Kodak) and quantitated using a Molecular Dynamics PhosphorImager SI and IMAGEQUANT 1.0 software. RNA from single-round, transcription reactions was recovered by electroelution from 8% polyacrylamide gels, extracted with phenol/chloroform and ethanol precipitated. Primer extension was used to map the start site of transcription as described earlier (26).

$KMnO_4$ Footprint Reactions. A 410-bp *Puv*II-*Bam*HI DNA fragment was isolated from pUCIIGtrpA after end-labeling of the template strand at the *Bam*HI site (27). Standard transcription reactions were prepared as described containing a final concentration of 4 nM labeled DNA. $KMnO_4$ modification (36) was carried out by the addition of 1 μl of a freshly prepared $KMnO_4$ solution (200 mM). After 3 min, reactions were terminated by the addition of 50 μl of stop solution (1.5 M 2-mercaptoethanol/0.1 mM EDTA/0.36 M sodium acetate, pH 7.0/120 μg/ml sonicated salmon testis DNA). The samples were ethanol precipitated, resuspended in 100 μl of 1 M piperidine, incubated 30 min at 90°C, and placed on ice. Samples were butanol precipitated (34) and residual piperidine was removed by vacuum centrifugation for 30 min. Samples were redissolved in 5 μl of formamide loading buffer and heated at 90°C for 2 min before loading onto an 8% polyacrylamide sequencing gel containing 7 M urea (34). An equivalent number of cpm from each reaction (10^5 cpm) was loaded. The gels were dried and exposed to x-ray film.

RESULTS

Spo0ABD and RNA Polymerase Induce DNA Strand Separation at the *spoIIG* Promoter. Previous studies in our lab have shown that the initial binding of *B. subtilis* RNA polymerase to the *spoIIG* promoter is not dependent on the presence of activated Spo0A (27). However, the complex formed by this binding initiates transcription at a very slow rate. Activated Spo0A accelerates the rate of initiation >50× as measured by formation of complexes that are resistant to the RNA polymerase inhibitor heparin (27) .

As another probe to determine the stage of initiation affected by activated Spo0A, we used potassium permanganate ($KMnO_4$) assays to monitor DNA strand separation (36). We showed earlier that the C-terminal fragment of Spo0A (termed Spo0ABD) was equivalent to Spo0A~P in its ability to stimulate *spoIIG* transcription (ref. 37; unpublished data). Because this protein eliminated the complication of the extent of phosphorylation, we used it to examine the structure of intermediate transcription initiation complexes by incorporating different sets of initiating nucleotides into the transcription reactions.

Ternary complexes containing end-labeled promoter DNA (26), 400 nM Spo0ABD, and 40 nM RNA polymerase were formed in the absence of nucleotide or under conditions that would allow formation of a dimer (ATP), trimer (ApA and GTP), or 11-mer (ATP and GTP) transcript. Samples were treated with $KMnO_4$ and after cleavage of the modified bases with piperidine, DNA fragments were separated on an 8% polyacrylamide gel containing 7 M urea (Fig. 1). Neither RNA polymerase nor Spo0ABD alone induced sensitivity of the promoter to $KMnO_4$. When added together, Spo0ABD and RNA polymerase induced a high level of sensitivity of the thymines between −3 and −13 (on the noncoding strand) relative to the start site of transcription, but the +1 site was not exposed. None of the thymines on the top (coding) strand were exposed under the same conditions (data not shown). We believe this lack of reactivity was due to the interaction of the coding strand with RNA polymerase (38–41), preventing modification of T residues in this region.

Because denaturation of the +1 site would be required for initiation of RNA synthesis, the complex containing −3 to −13 denatured in Fig. 1 does not represent a classical open complex, but rather an intermediate between closed and open complexes. Addition of ATP to the initiation reactions could lead to synthesis of the dinucleotide ApA, yet the addition of ATP, or GTP that we tested as a control, did not lead to accumulation of complexes with the +1 site exposed. In contrast, the addition of ApA and GTP led to exposure of the +1 and +2 sites on the template strand, indicating that initiated complexes could only accumulate when conditions allowed synthesis of at least a trimer. The thymines on the nontemplate strand remained unreactive (data not shown).

We reasoned that the nonreactivity of the thymines at +1 and +2 to $KMnO_4$ in the presence of Spo0ABD, RNA polymerase, and ATP was probably not due to protection of these residues by bound RNA polymerase as follows: formation of an ApApG trimer led to exposure of the thymine at +2, that would be two bases 5′ to the RNA polymerization site, suggesting that this site was available to $KMnO_4$. Incubation with ATP would place the thymine at +1 in the same relative position, two bases 5′ to the polymerization site. Because it was not modified, we concluded that it was not denatured. In the presence of ATP and GTP, the denatured region extended from −13 to +13 on the template strand (Fig. 1) and −20 to +17 on the coding strand (data not shown).

Transcription by RNA Polymerase from Heteroduplex Templates Replaces the Requirement for Activated Spo0A. If creation of the partial denaturation were the critical function of activated Spo0A, then efficient transcription from P*spoIIG* heteroduplex templates containing an artificial denatured bubble equivalent to that generated in the presence of Spo0ABD should be independent of Spo0ABD. Heteroduplex DNA templates were created by annealing single strand DNAs mutated to contain either the template or nontemplate strand sequence of the *spoIIG* promoter on both strands from position −7 to −14 or from −3 to −14. Five templates were used in the subsequent transcription assays and the sequences of the noncomplementary regions are shown in Fig. 2. Three major transcripts were observed from the heteroduplex templates by denaturing gel separation of the products of the transcription reactions. The RNAs were isolated and used as templates in primer extension experiments with the downstream primer used in the PCR reactions (IIG2). The major transcript produced from the wild-type, MB8NT, and MB12NT templates was initiated from the same nucleotide position as observed *in vivo* for the *spoIIG* promoter (data not shown). The start site for transcription from MB12T and MB8T was also the same as *in vivo*, despite the lower level of consensus of the −10 region to a σ^A promoter (5′-TATAtg-3′, matches are uppercase). Holoenzyme of *E. coli* also initiates transcription from a specific site adjacent to the edge of a DNA bubble (42–44). Two other transcription products smaller in size than the *spoIIG* wild-type transcript were also generated from the MB12T and MB8T templates. Neither of these transcripts produced a primer extension product when the downstream primer was used (data not shown). The sizes of these RNAs were compatible with the hypothesis that they were derived from transcription that occurred in the opposite direction, using the wild-type nontemplate strand as the template. For

the rest of this work, we concentrated on the major transcript, comparing the effects of reaction conditions and of Spo0A on its synthesis.

As expected, a low level of transcription was seen from the reannealed wild-type template in the absence of activated Spo0A (Fig. 3). Transcription increased significantly in the presence of Spo0A~P, or Spo0ABD, but not Spo0A. RNA polymerase alone produced more RNA from each of the heteroduplex templates than from the wild-type template (Fig. 3). Transcription from the template containing the 12 nucleotide mismatch bubble with the nontemplate sequence in the bubble region (MB12NT) was equivalent to that from wild-type templates in the presence of Spo0A~P. Reduction in the size of the bubble from 12 to 8 nucleotides caused a 75% decrease in the amount of transcript produced. Heteroduplex DNAs containing the template strand sequence of the promoter within the denatured region were not effective templates, regardless of the size of the mismatch region. We presume that the lower level of transcription reflected the lack of a −10 consensus sequence. This indicated that transcription from the denatured templates was still dependent on the sigma subunit. Slight increases over baseline levels were observed, in agreement with the reports of Aiyar *et al.* (43) that *E. coli* RNA polymerase will initiate transcription from heteroduplex templates lacking a sigma recognition sequence. The addition of Spo0A, Spo0A~P, or Spo0ABD had no effect on the level of transcript produced from any of the heteroduplex templates. The observation that the activated form of Spo0A did not stimulate transcription from the heteroduplex templates supports the idea that its effect on *spoIIG* transcription was restricted to the formation of the denatured regions.

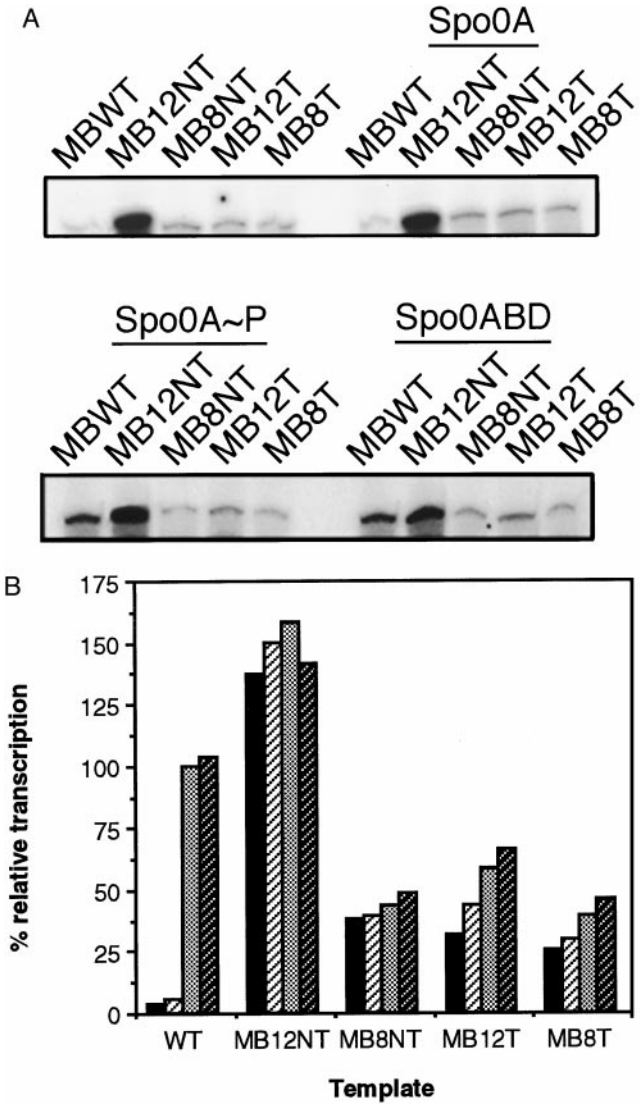

FIG. 3. Transcription from heteroduplex templates no longer requires activated Spo0A. (*A*) Wild-type or heteroduplex template DNA (4 nM, indicated above each lane) was used in transcription reactions with Spo0A, Spo0A~P, or Spo0ABD (400 nM). Reaction products were separated on a polyacrylamide gel as described in the *Materials and Methods* and the gel was exposed to x-ray film. The region of the film recording the major transcript is shown. (*B*) A graphic representation of the above results. The amount of transcript in each lane of the gel shown in *A* was quantitated by using a Molecular Dynamics PhosphorImager SI and IMAGEQUANT 1.0 and normalized to that produced from the wild-type template in the presence of Spo0A~P (100%); RNAP alone (solid columns), with Spo0A (white striped columns), Spo0A~P (gray columns) or Spo0ABD (gray striped columns).

The Presence of a Denaturation Bubble Removes a Barrier to Open Complex Formation at *spoIIG*. It has been suggested that the major enthalpy barrier to transcription initiation is related to denaturation of the DNA strands (reviewed in ref. 45). To test whether this barrier preceded the intermediate complex or not, we examined the temperature dependence of transcription from the homoduplex and heteroduplex templates over the range from 16°C to 42°C (Fig. 4).

Without Spo0ABD, transcription was not detected from the wild-type template below 30°C and above this temperature, transcription increased gradually with temperature. With MB8NT as the template, transcription was detected at 16°C and the level of transcription increased linearly with temperature up to 30°C. In contrast, a high level of transcription from MB12NT was observed over the entire temperature range, with levels from 7- to 10-fold greater than from wild-type and 4-fold greater than from MB8NT. Because Spo0A~P induces an 11-bp denaturation, this could account for the 8–10 fold increase in transcription from the wild-type promoter in its presence.

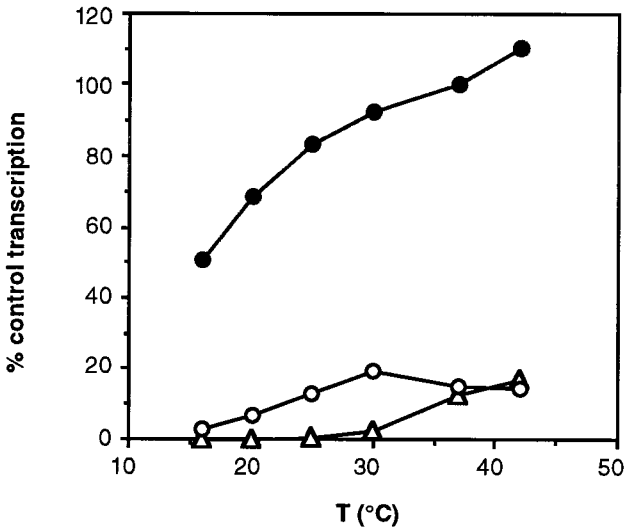

FIG. 4. The denaturation bubble removes a barrier to transcription from *spoIIG*. Reactions containing 4 nM template DNA (△, wild-type; ○, MB8NT; or ●, MB12NT), 80 mM potassium acetate, 40 nM RNA polymerase and the initiating nucleotides ATP and [α-^{32}P]GTP in transcription buffer were incubated at the indicated temperatures. After 2 min complexes were challenged with a mixture of UTP, CTP, and heparin. Products of the reactions were separated on a polyacrylamide gel as described in the *Materials and Methods*. The amount of transcript produced was quantitated by using a Molecular Dynamics PhosphorImager SI and IMAGEQUANT 1.0 software and normalized to that produced from the MB12NT template at 37°C (100%).

We also observed a temperature dependence for transcription from the heteroduplex templates. For both MB12NT and MB8NT, at 16°C only 50% of the level of transcript observed at 37°C was produced (Fig. 4). This result suggested an additional temperature effect that was not overcome by denaturation of the DNA strands between −3 and −13 and this may reflect the need to extend the denatured region to beyond the +1 site.

DISCUSSION

Changes in the length of the *E. coli* σ^{70} promoter spacer region decrease the efficiency of transcription initiation (45–47). This decrease in efficiency led to the hypothesis that the sigma factor binds to both −35 and −10 sequences to stimulate the DNA strand denaturation required for isomerization of closed to open complexes (48, 49). Kenney *et al.* (30) hypothesized that the length of the *spoIIG* spacer region (22 bp instead of the optimum 17 bp) prevents effective contact of the sigma subunit with the two consensus sequences and explains why transcription is low in the absence of Spo0A∼P. We have demonstrated that addition of activated Spo0A to a complex containing σ^A RNA polymerase and the *spoIIG* promoter, induced DNA strand denaturation in the −10 region of the promoter, but not at the +1 site. Furthermore, a modified *spoIIG* template containing a 12-bp denatured region upstream of the +1 site was transcribed at maximum rates in the absence of activated Spo0A and transcription was not stimulated by addition of activated Spo0A. We conclude that activated Spo0A cooperates with the RNA polymerase to induce the upstream denaturation and that stimulation of the *spoIIG* promoter by Spo0A occurs by accelerating this denaturation step.

Our model for Spo0A activation of the *spoIIG* promoter is as follows. Because a *spoIIG*-RNA polymerase complex can be demonstrated by electrophoretic mobility shift assays (M. A. Cervin, G.B.S., B. Raether, K. Ohlsen, M. Perego, and J. A. Hoch, unpublished information) and kinetic assays show that Spo0A∼P has no effect on the binding of the polymerase to the promoter (27), the first stage of the reaction is the binding of σ^A holoenzyme to the promoter without Spo0A. In DNase I protection assays RNA polymerase bound to the −35 region of the *spoIIG* promoter, and the −10 region was only slightly protected (27). This suggests that the barrier preventing transcription from the *spoIIG* promoter is the transition from the initial bound state to one where the sigma subunit can contact the −10 region.

The second stage of the initiation reaction is the binding of activated Spo0A to the RNA polymerase-*spoIIG* complexes. Because the 0A boxes overlap the −35 sequence of the *spoIIG* promoter, we propose that by contacting both the DNA and the sigma subunit, activated Spo0A allows sigma to release the −35 contacts without dissociation of the RNA polymerase from the DNA. The sigma subunit then repositions to contact the −10 sequence that results in protection of the −10 region, denaturation of the −3 to −13 region, and an increase in the rate of initiation.

In the third stage of the reaction, addition of ATP and GTP or ApA and GTP to the complex with the −13 to −3 denaturation leads to extension of the denatured region past +1 due to polymerization of the nascent transcript. The temperature dependence of transcription from wild-type templates in the presence of Spo0A∼P (unpublished data) is very similar to the dependence of transcription from the 12-bp bubble template in the absence of Spo0A∼P (Fig. 4), suggesting that Spo0A∼P did not facilitate the second denaturation step.

The *E. coli* regulator NtrC has also been found to activate transcription by stimulating DNA strand melting. Holoenzyme containing σ^{54} forms stable complexes at regulated promoters but cannot melt DNA in the absence of NtrC (31, 50–53) because the sigma factor contains a patch of amino acids that block its melting functions (32, 52–55). NtrC-P oligomers interact with σ^{54}, derepressing the melting function (55) and allowing conversion of closed to open complexes (52, 54). Other transcription regulators (for example, the phage lambda protein cI and the form of the cyclic AMP activating protein containing the AR3 mutation, see refs. 56 and 57 and the references therein) activate transcription by stimulating conversion of closed to open complexes through interaction with the sigma subunit. While experiments comparable to the ones we have reported have not been done with either regulator, most reports suggest that these proteins modify the activity of the sigma subunit in some manner, rather than repositioning it as we have suggested for Spo0A.

The proposed model for Spo0A activation of the *spoIIG* promoter assumes that the sigma subunit is relatively rigid and that activated Spo0A acts to lever it into position where it contacts the −10 sequence. Other models for the activity of Spo0A∼P are possible. For example, the MerR regulator binds to the *mer* operon promoter inducing a DNA deformation as evidenced by DNase I hypersensitive sites (58). The deformation may allow the sigma subunit to bridge the 19-bp distance between the −10 and −35 sequences at this promoter. This model for Spo0A seems unlikely to us because it does not induce dramatic DNA changes as measured by DNase I, hydroxyl radical, or $KMnO_4$.

Dombroski *et al.* (49), proposed that the −10 and −35 binding regions of the *E. coli* sigma subunit (regions 2.4 and 4.3, respectively) may interact, thus establishing an optimum spacer distance between the promoter consensus sequences. Transcription regulators could change the interaction of the two regions, allowing the sigma subunit to recognize promoters with unusual spacing. A model in which Spo0A induces sigma to contact both −35 and −10 sequences, implies that Spo0A and sigma would bind to the DNA simultaneously. At the *spoIIG* promoter, there are two 0A boxes (see Fig. 1), one of which overlaps the −35 consensus sequence completely. At the moment, we do not know whether both of the 0A boxes are filled when Spo0A activates transcription, although in all of the reported cases, two Spo0A proteins appear to bind DNA in tandem, at adjacent sites (25, 59). The hydroxyl radical and DNase I footprints of Spo0A∼P binding show that it binds in the DNA minor groove 5′ to the 0A box and on one face of the helix within the 0A box (59, 60). Thus it is possible that Spo0A and sigma could contact the 0A box/−35 sequence simultaneously and we cannot rule out the sigma modification model. However, it does seem simpler to assume that only one protein binds to the −35 region at a time. Direct genetic evidence for Spo0A-σ^A interactions has been obtained because mutations of sigma have been described that block activation of *spoIIG*, but do not affect expression of other promoters (61, 62). These mutations are in region 4 of the sigma subunit and are likely to be spatially close to Spo0A∼P when it is bound at the 0A boxes.

A striking feature of the data in Fig. 1 is that the denaturation of the *spoIIG* promoter occurred in two steps: positions −13 to −3 were denatured first without initiating nucleotides and then the denaturation extended beyond the +1 site in the presence of nucleotides. This denaturation conforms to a "melting from the edge" pattern, because denaturation of the +1 site begins distal to that position (45). This pattern has been reported for other promoters, for example the *Bacillus* promoter, *trnS,* also shows nucleation of denaturation in the −10 region of the promoter (63). Our interpretation is that the two-step denaturation is not a necessary component of regulation by Spo0A∼P, although the *spoIIG* promoter provides a dramatic example of this process. The separation of the two steps may provide a useful experimental tool to dissect the

mechanism behind DNA melting that, as has been pointed out (56, 57), is one of the mysteries of transcription initiation.

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