3806–3812 *Nucleic Acids Research, 1998, Vol. 26, No. 16*

The *Bacillus subtilis* regulator SinR inhibits *spoIIG* promoter transcription *in vitro* without displacing RNA polymerase

Marguerite A. Cervin[1], Richard J. Lewis[3], James A. Brannigan[3] and George B. Spiegelman[1,2,*]

[1]Department of Microbiology and Immunology and [2]Department of Medical Genetics, University of British Columbia, Vancouver, BC V6T 1Z3, Canada and [3]Department of Chemistry, University of York, Heslington, York YO1 5DD, UK

Received March 19, 1998; Revised June 22, 1998; Accepted June 30, 1998

ABSTRACT

Initiation of sporulation in *Bacillus subtilis* is controlled by several regulators which affect activation by phosphorylation of the key response regulator Spo0A or transcription of Spo0A~P-dependent genes. *In vivo* overexpression of one of these regulators, *sinR*, results in suppression of transcription from the Spo0A~P-dependent promoters of *spo0A*, *spoIIA*, *spoIIE* and *spoIIG* and *in vitro* SinR binds to the promoters of the *spoIIA* operon and the *spo0A* gene. In this study we have demonstrated that *in vitro* SinR directly repressed Spo0A~P-dependent transcription by *B.subtilis* RNA polymerase from the *spoIIG* operon promoter. SinR inhibited transcription prior to formation of heparin-resistant complexes but did not displace RNA polymerase from the *spoIIG* promoter. DNase I protection studies demonstrated that SinR protected a large region of the *spoIIG* promoter and induced DNase I hypersensitive sites, particularly around the 0A boxes, at the same positions as those induced by zinc. Since binding of zinc induces bends in the DNA, we concluded that SinR binding also altered the conformation of the *spoIIG* promoter. We propose that SinR-induced conformational changes in Spo0A-dependent promoters prevent activation of transcription by Spo0A~P.

INTRODUCTION

The initiation of sporulation in *Bacillus subtilis* is dependent on activation of the response regulator Spo0A by phosphorylation via the multi-component phosphorelay (1,2). Binding of phosphorylated Spo0A (Spo0A~P) to specific DNA binding sites (0A boxes) (3,4) can either repress or activate transcription depending on the nature of the promoter and the position of the 0A box relative to the transcription start site (5,6). A series of checkpoints, in the form of negative regulators, control both phosphorylation of Spo0A and the transcription activation properties of Spo0A~P. The flow of phosphate through the phosphorelay is regulated by the activity of sensor kinases (KinA, KinB or KinC), which control phosphate input, and phosphatases, which control phosphate removal from a specific phosphorelay component (7,8).

While it is clear that phosphorylation of Spo0A is required for sporulation, other regulators have been described which do not appear to act by affecting the level of Spo0A~P. We have recently shown that Spo0JA/Soj inhibits Spo0A~P-dependent activation of transcription of the stage II sporulation promoter *spoIIG* by dissociating the Spo0A~P–RNA polymerase–DNA complex (9). Another regulator, *sinR*, represses sporulation by preventing expression of the Spo0A~P-dependent stage II sporulation genes *spoIIA*, *spoIIG* and *spoIIE in vivo* when introduced into *B.subtilis* on a multicopy plasmid (10,11). SinR is a multifunctional protein which, besides repressing sporulation, also regulates genes for motility (12,13), alkaline protease expression (*aprE*) and competence development (14).

SinR is a 14 kDa, dimeric DNA-binding protein. It is a member of the Cro family of transcription regulators and has a predicted H-T-H–leucine zipper–H-T-H structure (15,16). Regulation of SinR activity occurs at the post-translational level and is carried out by the protein product of *sinI*, the other gene in the *sin* operon. While expression of *sinR* is fairly constant throughout the growth cycle, the expression of *sinI* is stimulated by sporulation activators Spo0A~P and σ^H and inhibited by sporulation repressors AbrB, Hpr and glucose. SinI interacts with the C-terminal end of SinR, disrupting the dimer and therefore preventing SinR from binding to DNA (16,17).

The mechanism for SinR repression of transcription has not been established. DNase I and DMS footprint assays indicate that SinR binds near the promoters it represses (*aprE*, *spo0A* and *spoIIA*; 11,14,15). In the case of the *aprE* promoter SinR protects a 46 bp sequence, containing a region with dyad symmetry, at –220 to –265 relative to the start site of transcription (15). The SinR-protected regions at the promoters for the *spoIIA* operon and the *spo0A* gene include the 0A boxes (11,14), which may be critical for induction of these promoters by Spo0A~P. These findings suggest that SinR could inhibit transcription by competing with Spo0A~P for binding to DNA, although it could act by a different mechanism at the *aprE* promoter. We have undertaken

*To whom correspondence should be addressed. Tel: +1 604 822 2036; Fax: +1 604 822 6041; Email: spie@unixg.ubc.ca

an *in vitro* study of repression of Spo0A~P-dependent transcription by SinR using the well-characterized Spo0A~P-dependent promoter of the *spoIIG* operon.

MATERIALS AND METHODS

Purified proteins and plasmids

The σ^A RNA polymerase was purified from *B.subtilis* as previously described (18). Purified Spo0A, Spo0F, Spo0B and KinA were obtained from Dr James Hoch (Scripps Institute, La Jolla, CA). SinR was purified as described previously (16). The protein was 98% pure and has been used for structure determination. Two preparations of SinR were tested with identical results.

The *spoIIG* template DNA used in all the assays was isolated from the plasmid pUCIIGtrpA (19). This plasmid has 100 bp of *B.subtilis* DNA upstream of the *spoIIG* transcription start site (+1), including the Site 1 and Site 2 0A boxes (Site 1 at –82 to –97; Site 2 at –37 to –56), 130 bp downstream of +1 and the *trpA* transcription terminator. All chemicals were obtained from Sigma unless otherwise stated; restriction enzymes and T4 polynucleotide kinase were obtained from Gibco BRL.

In vitro transcription assays

The *spoIIG* template DNA used for the *in vitro* transcription assays was isolated from pUCIIGtrpA as a 600 bp *Pvu*II fragment containing the *spoIIG* promoter, 130 bp downstream of the +1 transcription start site, the *trpA* terminator and ~100 bp of vector sequences both upstream and downstream of *spoIIG* DNA.

Single round *in vitro* transcription assays (20,21) were carried out in 1× transcription buffer (0.01 M HEPES, pH 8.0, 0.01 M MgAc, 1 mM DTT, 0.1 mg/ml BSA, 80 mM KAc) in a final volume of 10 µl. Phosphorylation of Spo0A was carried out using the *in vitro* phosphorelay reaction exactly as previously described (1,20). RNA polymerase (25 nM) was incubated with Spo0A~P (indicated input), template DNA (2 nM) and the initiation nucleotides ATP (0.4 mM) and [α-^{32}P]GTP (0.005 mM, 20 Ci/mM; NEN) for 3 min at 37°C. This was followed by an elongation step brought about by addition of UTP, CTP (0.4 mM each) and heparin (10 µg/ml) and a 5 min incubation at 37°C. The reactions were terminated by addition of 5 µl stop buffer (7 M urea in 2× TBE) (1× TBE is 0.09 M Tris–borate, 0.002 M EDTA; 21) and 0.1% of each of the tracking dyes bromophenol blue and xylene cyanol. SinR was added to the assays where indicated. The 130 nt transcripts were separated by electrophoresis through an 8% polyacrylamide gel (40:1.38 acrylamide:*N*,*N*′-methylene bis-acrylamide; BioRad) for 30 min at 600 V. Transcripts were detected by exposure to X-ray film (Kodak XAR) overnight at –80°C and by the Molecular Dynamics SI PhosphorImaging system. RNA polymerase was diluted in 1× transcription reaction buffer containing 10% glycerol, Spo0A~P was diluted in 1× transcription buffer and SinR was diluted in SinR dilution buffer (0.01 M HEPES, pH 8.0, 250 mM NaCl, 2% glycerol).

Electrophoretic mobility shift assays

The DNA fragment used for the electrophoretic mobility shift assays (EMSA) was the 230 bp *Bam*HI–*Hin*dIII fragment from pUCIIGtrpA, which contains only *spoIIG* sequences, that had been end-labeled at the *Bam*HI site with [γ-^{32}P]ATP (7000 Ci/mmol; ICN Biochemicals) and T4 polynucleotide kinase. The labeled fragment was recovered by electroelution after electrophoresis through a 4% non-denaturing polyacrylamide gel. The eluted fragment was ethanol precipitated and resuspended in TEA (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 20 mM sodium acetate).

We tried several different buffer conditions in an attempt to obtain a consistent DNA mobility shift with SinR. Initially, we tested the same conditions as those described for the transcription assays, 1× transcription buffer and 3–8 min incubation periods. These conditions were sufficient for formation of SinR–RNA polymerase–*spoIIG* promoter complexes, but SinR did not bind consistently on its own. Since Mandic-Mulec *et al*. (11) and Bai *et al*. (17) have reported gel mobility shifts with SinR at the *aprE* and *spo0A* promoters, we used a buffer similar to theirs, 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mg/ml BSA, 1 mM β-mercaptoethanol and 10% glycerol. However, in spite of trying various additions to this buffer, including spermidine and calf thymus DNA, incubating SinR with the *spoIIG* fragment for varying lengths of time, up to a maximum of 30 min, and at varying temperatures, including 37, 30 and 25°C, and with varying glycerol concentrations, we did not get reproducible binding of SinR. Finally, we tried adding various metals, including zinc acetate (ZnAc), manganese chloride and calcium chloride. The addition of ZnAc (minimum concentration 0.2 mM) resulted in a consistent DNA mobility shift in the presence of SinR when the reactions were incubated for 15 min at 37°C and EDTA was eliminated from the electrophoresis buffer. Reactions were stopped by addition of 3 µl stop buffer (1× gel shift reaction buffer, 2% glycerol, 0.3 µg/ml sonicated calf thymus DNA) and immediately loaded onto a non-denaturing polyacrylamide gel (40% acrylamide, 1× Tris–borate buffer) running at 20 mA in 1× Tris–borate (0.09 M Tris–HCl, 0.09 M borate). Electrophoresis was carried out for ~3 h, the gels were dried and then exposed to X-ray film overnight at –80°C. The 1× reaction buffer for the EMSA contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mg/ml BSA, 1 mM β-mercaptoethanol, 5% glycerol. SinR–DNA complex formation was tested using several inputs of both zinc (0, 0.1, 0.2, 0.3, 0.4, 0.8, 1 and 10 mM) and SinR (250, 500, 750 and 1000 ng). Only three SinR–DNA complexes were formed and representative inputs of zinc and SinR (indicated in the figure legends) that demonstrated these complexes were used for the assays shown in Figure 3.

DNase I footprint assays

DNase I footprint reactions were done essentially as described previously (20). The DNA template was a 410 bp *Bam*HI–*Pvu*II fragment from pUCIIGtrpA end-labeled at the *Bam*HI site as described above. DNA (2 nM) was incubated with the indicated input of SinR or SinR dilution buffer in 1× gel shift reaction buffer, with or without 0.2 mM ZnAc, in a final volume of 20 µl for 15 min at 37°C. The samples were then treated with DNase I (final concentration 1.3 µg/ml) for 10 s and stopped by addition of 3 vol stop buffer (0.1% SDS, 4. 0 mM EDTA, 270 mM sodium chloride and 40 µg/ml sonicated calf thymus DNA). The samples were ethanol precipitated and resuspended in 5 µl formamide loading buffer (95% formamide in 2× TBE, 1% bromophenol blue, 1% xylene cyanol). After boiling the samples for 3 min, an equal amount of radioactivity from each sample was loaded onto an 8% polyacrylamide sequencing gel containing 7 M urea. The samples were electrophoresed at 45 W for ~4 h, the gels dried and then exposed to X-ray film overnight at –80°C. To determine the

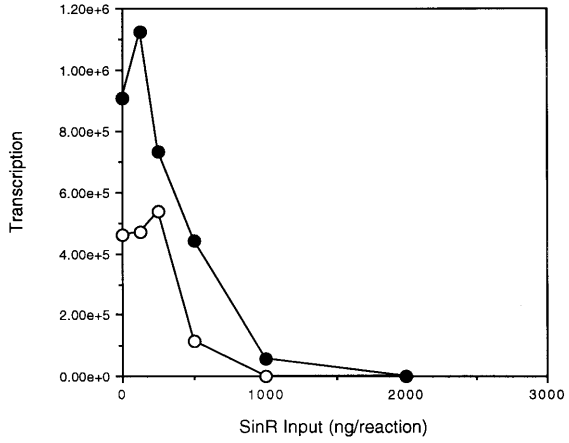

Figure 1. SinR inhibits Spo0A~P-dependent *spoIIG* transcription. *Bacillus subtilis* RNA polymerase was incubated with the *spoIIG* promoter for 3 min at 37°C followed by addition of the indicated input of SinR (or 1 µl SinR dilution buffer). After a further 5 min incubation, Spo0A~P, ATP and GTP were added followed by a 3 min incubation. Complexes of RNA polymerase and DNA which had initiated RNA synthesis were allowed to elongate by the addition of heparin, UTP and CTP. Reactions were stopped and transcripts separated by electrophoresis through an 8% denaturing polyacrylamide gel. Spo0A~P input 200 nM (open circles); Spo0A input 400 nM (closed circles). The transcription assays were analyzed using the Molecular Dynamics PhosphorImaging system and ImageQuant software. The levels of transcription (*y*-axis) are presented in arbitrary units generated by the PhosphorImaging software.

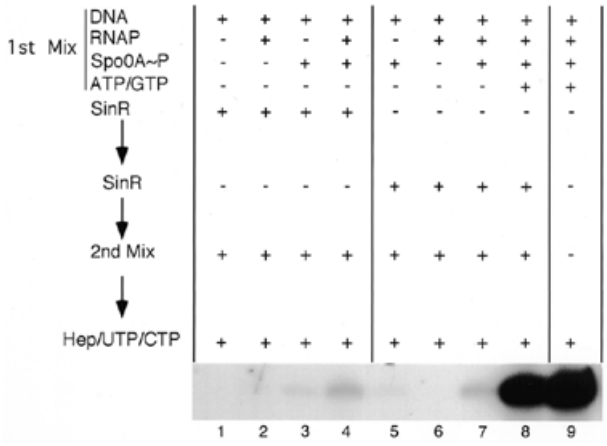

Figure 2. SinR inhibits *spoIIG* transcription prior to formation of an initiated complex. This Figure shows an autoradiograph detecting the production of full-length transcripts when SinR was added either with the indicated components (lanes 1–4) or after preincubation of the indicated components (lanes 5–8). The level of transcription in the absence of SinR is shown in lane 9. The first mix is comprised of all of the components needed for synthesis of the initial 11 base RNA and conversion of the RNA polymerase from a heparin-sensitive to a heparin-resistant complex. In each case, the second mix contains components of the first mix that were not added in the first step. Each incubation step was carried out for 3 min at 37°C, except for the elongation reaction (addition of heparin/UTP/CTP) which was a 5 min incubation at 37°C. SinR was added at an input of 1000 ng. Each reaction contained a Spo0A~P input of 200 nM. Lane 9, no SinR added.

nucleotide positions relative to the +1 transcription start site, the end-labeled DNA fragment was digested with *Hin*dIII (–100), *Ase*I (–43) or *Alu*I (–27) to produce size markers and electrophoresed in lanes adjacent to the footprint reactions. A reaction containing only Spo0A~P was used to identify the Spo0A binding sites.

RESULTS

SinR inhibits Spo0A~P-dependent *spoIIG* transcription prior to formation of an initiated complex

To examine the mechanism of SinR regulation we first determined its overall effect on *in vitro* transcription from the Spo0A~P-activated, σ^A-dependent *spoIIG* promoter (23,24). Figure 1 shows the results from a representative assay in which SinR inhibition of transcription was tested in the presence of two inputs of Spo0A~P. SinR directly repressed Spo0A~P-dependent transcription. Transcription by 200 or 400 nM Spo0A~P was inhibited by 50% at SinR inputs of 400 or 500 ng respectively.

We have shown previously that transcription initiation at the *spoIIG* promoter occurs by the following general pathway. First, RNA polymerase binds rapidly and reversibly to the promoter region. Second, Spo0A~P binds to the complex of promoter and RNA polymerase. Third, conformational changes occur which permit initiation of RNA synthesis. In the presence of ATP and GTP, the RNA polymerase can synthesize an 11 base RNA, but it does not leave the promoter at this step. Transition to elongation occurs as the RNA chain length increases between 11 and 16 bases (20,25).

To determine whether transcription became resistant to SinR inhibition at any step, we monitored the ability of SinR to inhibit production of full-length transcripts when added to pre-mixed combinations of Spo0A~P, RNA polymerase and DNA. After a brief incubation, mixtures were supplemented with all the components which would allow formation of initiated complexes and, after a further incubation, the level of initiated complexes formed was monitored by allowing a single round of transcript elongation (Fig. 2). When SinR was added to mixtures which contained template DNA, Spo0A~P, RNA polymerase, ATP and GTP, the level of transcript produced was the same as the control (compare lanes 8 and 9). Since these conditions allowed formation of initiated complexes, SinR did not block elongation of RNA synthesis.

In contrast, addition of SinR at any step prior to initiation reduced transcription dramatically (lanes 1–7), so SinR must block initiation. There were slight variations in transcription levels with certain combinations. For example, incubation of template DNA, RNA polymerase and Spo0A~P alone (lane 7) or with SinR (lane 4) slightly raised the level of initiated complexes. This suggested that the complex of RNA polymerase and Spo0A~P resisted SinR inhibition, which in turn suggested that SinR may compete with Spo0A~P for binding. However, SinR was able to completely inhibit transcription when added either before (lane 1) or with RNA polymerase (lane 2). Thus SinR could have displaced either the RNA polymerase or Spo0A~P from the promoter or bound with the RNA polymerase–DNA complex and inhibited transcription activation by Spo0A~P.

SinR binds and induces conformation changes at the *spoIIG* promoter

Since SinR has been shown to bind at the promoters for other Spo0A~P-dependent genes (11,14,15), we examined binding of SinR at the *spoIIG* promoter using EMSA and DNase I footprint

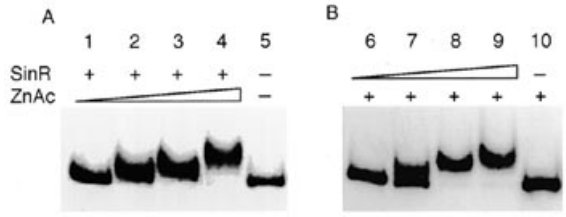

Figure 3. SinR binds to the *spoIIG* promoter in the presence of ZnAc. (**A**) An autoradiograph of an EMSA in which SinR was incubated with 2 nM labeled template for 15 min at 37°C in 1× gel shift reaction buffer containing ZnAc. Reactions were terminated, the samples were electrophoresed through a 6% non-denaturing polyacrylamide gel in 1× TB electrophoresis buffer, the gels dried and the SinR–*spoIIG* complexes detected by autoradiography. Lanes 1–4, 500 ng SinR + *spoIIG* in buffer containing 0.1, 0.2, 0.3 or 0.8 mM ZnAc respectively; lane 5, free DNA (without ZnAc). (**B**) Increasing inputs of SinR were incubated with the end-labeled *spoIIG* template for 15 min at 37°C in gel shift reaction buffer with 0.2 mM ZnAc and then loaded onto a non-denaturing gel as described in (A). Lanes 6–9, SinR inputs of 125, 250, 500 and 1000 ng respectively; lane 10, DNA + 0.2 mM ZnAc.

assays. In spite of its similarity to other DNA-binding proteins, we had great difficulty obtaining a consistent DNA mobility shift with SinR under conditions used for transcription. Therefore, we explored conditions that would allow formation of a SinR–*spoIIG* promoter complex that was stable to electrophoresis. After testing several different conditions (see Materials and Methods) we found that addition of zinc acetate (ZnAc) to the reaction buffer at a minimum final concentration of 0.2 mM resulted in a consistent SinR-dependent DNA shift. Even under these conditions, maximum binding required a SinR–*spoIIG* promoter incubation period of 15 min at 37°C. Figure 3A shows an EMSA with a constant input of SinR incubated with an end-labeled *spoIIG* fragment in the presence of increasing inputs of ZnAc. Three SinR–DNA complexes with different mobilities were formed with ZnAc inputs of up to 0.8 mM (Fig. 3A, lanes 1–4). There were no further effects on SinR–DNA complex formation until 10 mM ZnAc, at which point complex formation began to be inhibited (data not shown). Complexes with similar mobilities were formed when the ZnAc input was constant and the SinR input increased (Fig. 3B, lanes 6–9). Thus the complexes most likely represented loading of SinR onto the promoter. The inclusion of other metals, such as magnesium, calcium or manganese did not affect the ability of SinR to shift the DNA in these assays. The reaction and electrophoresis buffers used for these assays lacked EDTA. The inclusion of EDTA in the electrophoresis buffer resulted in dissociation of the complexes formed, even in the presence of 1 mM ZnAc.

Since the amino acid sequence of SinR did not suggest the presence of zinc fingers (26) and zinc has been shown to bind to DNA at specific sequences (27) and induce bends (28,29), we hypothesized that zinc was binding to and affecting the DNA such that SinR was able to bind and maintain a stable complex. Indeed, there are several sequences on the template strand of *spoIIG* which are potential zinc binding sites (27): TGGGA, GG and GGG at positions –27, –55 and –72 relative to the transcription start site (+1) respectively.

To investigate the ZnAc effect and define the SinR binding region on *spoIIG* we used DNase I protection analysis. SinR was incubated with the *spoIIG* fragment in the absence or presence of ZnAc for 15 min, followed by treatment with DNase I and

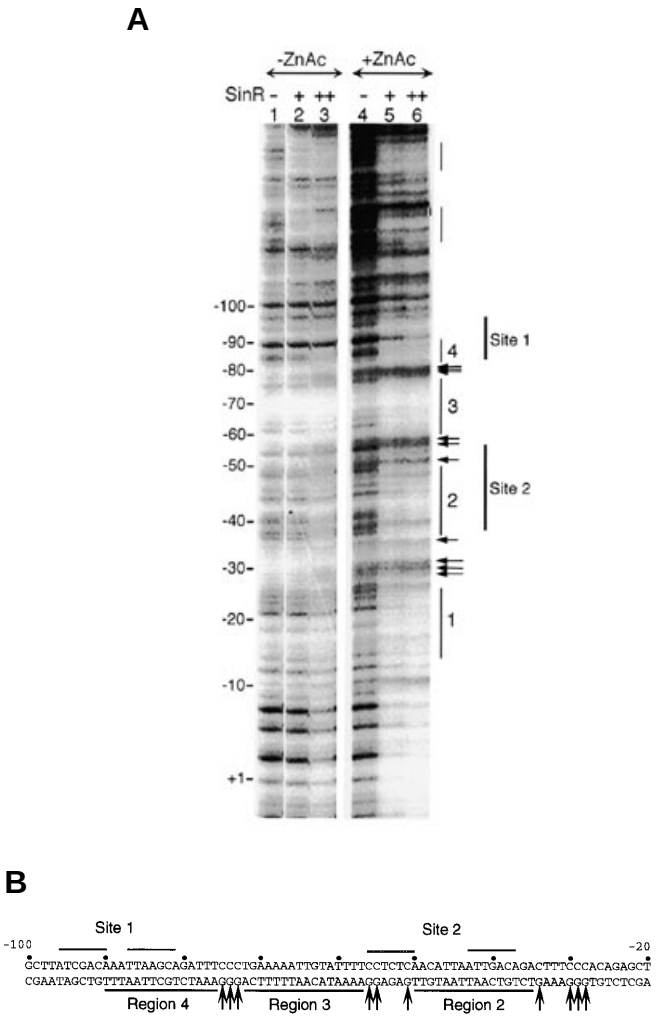

Figure 4. SinR binding induces conformational changes in the *spoIIG* promoter. (**A**) An autoradiograph of a DNase I footprint analysis of the SinR binding site at the *spoIIG* promoter. The end-labeled *spoIIG* fragment was incubated with SinR for 15 min in reaction buffer (± 0.2 mM ZnAc) at 37°C for 15 min, followed by treatment with DNase I. An equal amount of radioactivity was loaded onto an 8% polyacrylamide gel containing 8 M urea and processed as described in Materials and Methods. The numbers on the left-hand side indicate the nucleotide distance relative to the transcription start site (+1); thick vertical lines denote the Site 1 and Site 2 0A boxes; thin vertical lines indicate the SinR-protected regions; arrows indicate the DNase I HS sites. The unlabeled vertical lines represent the SinR-protected regions in the vector sequences. Reactions in lanes 1 and 4, without SinR, contained SinR dilution buffer. SinR was added to reactions at an input of 250 (+) or 500 (++) ng. (**B**) The nucleotide sequence of the *spoIIG* promoter from –20 to –100, relative to the +1 transcription start site (19), with the locations of the SinR DNase I-protected regions (black lines below the diagram) and HS sites (arrows) from (A) marked. The positions of the Site 1 and Site 2 0A boxes are indicated above the diagram.

separation of the DNA fragments by electrophoresis. Figure 4A shows an autoradiograph of the products of the DNase I protection assay. The nucleotide positions relative to the transcription start site (+1) and the positions of the Site 1 and Site 2 0A boxes are indicated.

Incubation of the *spoIIG* promoter with 0.2 mM ZnAc resulted in formation of DNase I hypersensitive (HS) sites, indicated by arrows, that were not formed when the DNA was treated without

ZnAc (compare lanes 4 and 1). When SinR was incubated with the *spoIIG* promoter in the presence of ZnAc, the HS sites were still formed and four regions between the HS sites became protected (lanes 5 and 6): –13 to –27, –35 to –50, –57 to –72 and –76 to –90 (thin lines). HS sites mapped to multiple guanosine residues (GGG, –28 to –30; GG, –55, –56; GGG, –73 to –75) or single guanosine residues (–34 and –51).

In the absence of ZnAc, the DNase I protection pattern induced by SinR was less obvious, but the same regions were protected with the exception of region 1 (–13 to –27). With a SinR input of 500 ng, most of the HS sites were formed that were noted in the reactions with ZnAc. Two HS sites (–38 and –48) that were detected in the presence of ZnAc were absent in reactions without ZnAc but containing SinR and, for clarity, these sites have not been marked on the figure. We therefore concluded that SinR had two effects on the *spoIIG* promoter, protection of three regions and induction of DNase I HS sites at some of the positions induced by incubation of *spoIIG* with ZnAc. Zinc has been shown to induce severe bends or kinks (bend angles >45°; 27,28) in DNA, so it is likely that binding of SinR also induces bends in DNA. The SinR-dependent DNase I HS sites and protected regions are mapped on the DNA sequence of the *spoIIG* promoter shown in Figure 4B. Since the SinR-induced DNase I HS sites are located around the Site 2 0A box, the conformational changes may contribute to inhibition of Spo0A~P stimulation of transcription.

SinR also protected an upstream region of the DNA fragment beyond the sequences derived from the *spoIIG* promoter, which end at a *Hin*dIII site at –100 (Fig. 4A, unmarked vertical lines). By comparing the mobility of the DNA fragment used in the DNase I assay after digestion with *Hin*dIII, *Msp*I and *Hha*I (the latter two cut only within the vector sequences on the fragment) and the position of the upstream SinR footprint, we determined that these sites were in the *lac* operator sequence located adjacent to the multiple cloning site in the vector. To ensure that these sites were not involved in SinR inhibition of *spoIIG* promoter transcription, we tested transcription from the 230 bp *Hin*dIII–*Bam*HI fragment of pUCIIGtrpA, which includes the *spoIIG* sequences but not the flanking vector sequences. The effect of SinR on Spo0A~P-dependent transcription from both the 600 bp *Pvu*II and *Hin*dIII–*Bam*HI templates was identical, confirming that the upstream SinR binding sites in the vector sequences were not involved in SinR inhibition of *spoIIG* transcription (data not shown).

SinR does not displace RNA polymerase from the *spoIIG* promoter

While the data in Figures 2–4 showed that SinR bound to the *spoIIG* promoter and inhibited transcription before the step of RNA synthesis initiation, the question remained whether or not SinR prevented binding of RNA polymerase to the promoter. To answer this question we used EMSA, to monitor complex

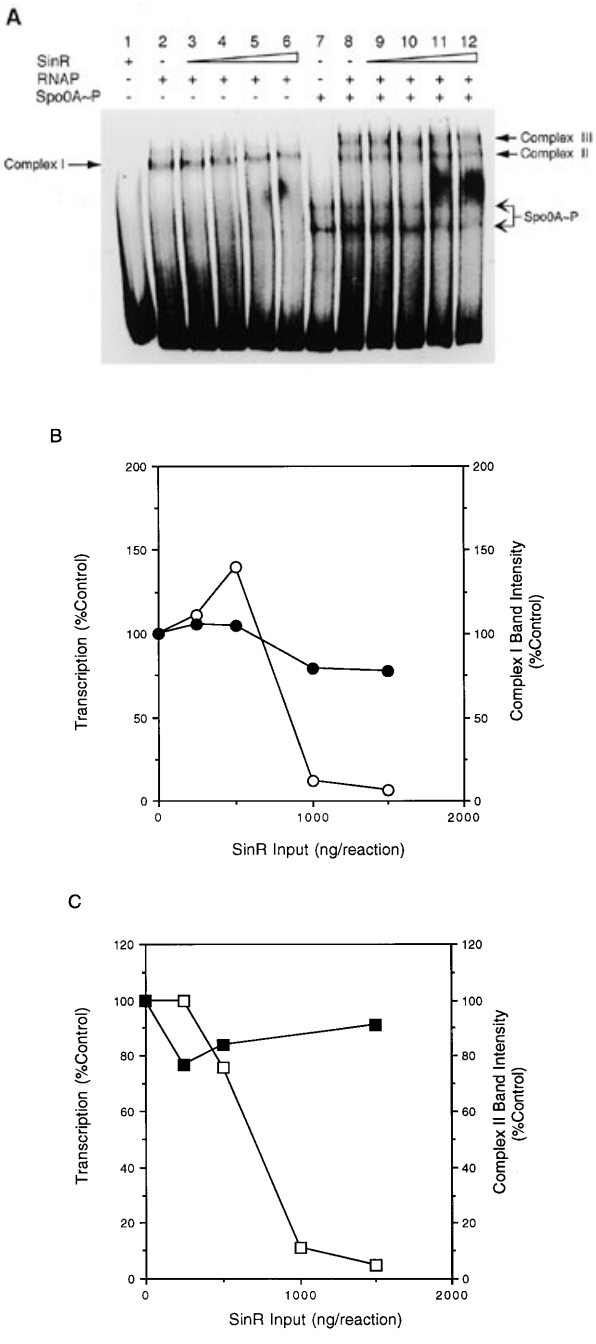

Figure 5. SinR does not displace RNA polymerase from the *spoIIG* promoter. (**A**) An autoradiograph of an EMSA in which 2 nM end-labeled *spoIIG* template was incubated with SinR (or SinR dilution buffer), RNA polymerase, Spo0A~P or RNA polymerase and Spo0A~P for 3 min at 37°C in 1× gel shift reaction buffer followed by addition of increasing inputs of SinR and a further 5 min incubation. The reaction was stopped by addition of a non-competitive inhibitor, calf thymus DNA, and immediately loaded onto a 5.2% non-denaturing polyacrylamide gel. Following electrophoresis, the gels were dried and exposed to X-ray film. Lane 1, SinR (1500 ng); lane 2, RNA polymerase (25 nM); lanes 3–6, RNA polymerase + 500, 750, 1000 or 1500 ng SinR respectively; lane 7, Spo0A~P (400 nM); lane 8, RNA polymerase + Spo0A~P (400 nM); lanes 9–12, RNA polymerase + Spo0A~P (400 nM) + 500, 750, 1000 or 1500 ng SinR respectively. Positions of complexes formed by RNA polymerase (complex I), Spo0A~P or RNA polymerase–Spo0A~P (complexes II and III) are indicated on the Figure. (**B** and **C**) Transcription assays were carried out under the conditions used in the EMSAs shown in (A) and plotted with the relative band intensities of complex I (B) or complex II (C) from (A). Spo0A~P was added at an input of 400 nM. The level of transcription and relative intensities of the bands formed by the complexes shown in (A) were determined using the Molecular Dynamics PhosphorImager and ImageQuant software. The results are expressed as a percentage of the control reactions without SinR. (B) Plot of the relative band intensities of complex I (A, lanes 2–6), closed circles; plot of transcription activity, open circles. (C) Plot of the relative band intensities of complex II (A, lanes 8–12), closed squares; plot of transcription activity, open squares.

formation, and the corresponding transcription assays, to determine transcription activity. Figure 5A shows an EMSA in which RNA polymerase or RNA polymerase and Spo0A~P were incubated with the end-labeled *spoIIG* promoter followed by an incubation with increasing inputs of SinR. Using these assay conditions, SinR–DNA complex formation was variable (lane 1). RNA polymerase bound to the DNA and resulted in formation of a single complex (lane 2, complex I). Addition of SinR resulted in two changes to complex I. First, there was a slight loss of the complex (as seen by the decrease in band intensity). Second, the mobility of the complex decreased as the SinR input increased (lanes 3–6). These results suggest that SinR bound to the DNA–RNA polymerase complex without displacing the RNA polymerase. Spo0A~P formed two complexes with the DNA (lane 7). Spo0A~P and RNA polymerase also formed two complexes (complex II and complex III) at the *spoIIG* promoter (lane 8). As was the case with complex I, increasing amounts of SinR did not displace either complex II or III (lanes 9–12).

To compare the slight decrease in the DNA–RNA polymerase complexes with SinR-mediated inhibition of transcription, transcript production was monitored under the conditions used in the EMSAs. Figure 5B and C shows the transcription levels and the changes in relative band intensities of complexes I and II respectively as a function of SinR input. Addition of SinR after incubation of either RNA polymerase (Fig. 5B) or RNA polymerase and Spo0A~P (Fig. 5C) with the DNA resulted in 90% inhibition of transcription but only 20% displacement of either complex I or complex II. Results for complex III were similar to those for complex II (data not shown). Thus, SinR inhibition could not be explained by blocking RNA polymerase access to the promoter. It was impossible to determine from the mobility of complexes II and III whether or not Spo0A~P was retained in the complex as the input of SinR increased or whether SinR displaced Spo0A~P. Experiments aimed at showing Spo0A~P release by western blot analysis or by using [γ-^{32}P]ATP-labeled Spo0A~P proved impossible, because in the native gel system which allowed detection of the effect of SinR, free Spo0A~P migrated at the same position as the DNA–RNA polymerase complexes.

If SinR inhibited transcription by competing with Spo0A~P, then possibly Spo0A~P could overcome SinR inhibition. To test this idea, we added increasing inputs of Spo0A~P to two sets of transcription assays: one without SinR and one where SinR and RNA polymerase were preincubated with the *spoIIG* promoter (Fig. 6). After an initial lag, transcription from the *spoIIG* promoter was restored to control levels by increasing the input of Spo0A~P. These results suggested that SinR was displaced by Spo0A~P and that the presence of SinR altered Spo0A~P binding or activation.

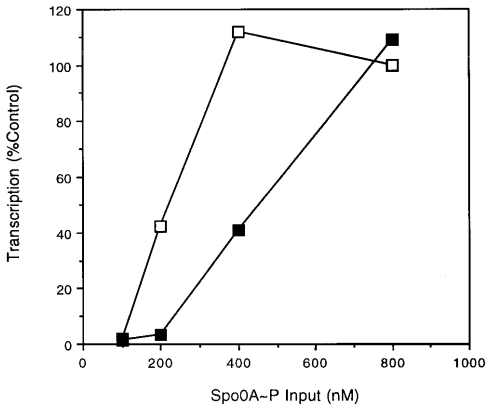

Figure 6. Spo0A~P overcomes SinR inhibition of transcription. SinR or SinR dilution buffer was added to transcription assays after binding of RNA polymerase to the DNA. Spo0A~P was then added at an input of 100, 200, 400 or 800 nM and after a 3 min incubation the initiation nucleotides ATP and GTP were added. The initiated complexes were allowed to elongate by the addition of UTP, CTP and heparin and the level of transcription determined as described in the legend to Figure 1. Control reaction without SinR, open squares; reactions containing SinR (500 ng/reaction), closed squares. The results are presented as a percentage of the maximum level of transcription in the control reaction.

DISCUSSION

Epistasis experiments suggest that SinR is a multifunctional regulator capable of activating and suppressing alternative pathways, such as competence and sporulation in *Bacillus* (10,11,14). The mechanism by which SinR activates competence remains obscure, but it was proposed that SinR repressed sporulation by inhibition of transcription from the *spo0A* and three key stage II operon promoters (11). In this study we have demonstrated that SinR directly repressed transcription of one of these promoters, the *spoIIG* operon promoter, *in vitro*. The order of addition experiments shown in Figure 2 demonstrated that SinR acted before formation of heparin-resistant complexes. As heparin resistance at the *spoIIG* promoter is reached after synthesis of two phosphodiester bonds (D.A.Rowe-Magnus and G.B.Spiegelman, unpublished results), SinR inhibition must occur before this stage.

EMSAs with RNA polymerase alone or with Spo0A~P present indicated that SinR did not displace RNA polymerase from the promoter. The direct effect on Spo0A~P binding was more difficult to interpret, since the possibility that binding of SinR replaced Spo0A without altering the mobility of the complex was not resolved. Thus, two mechanisms for SinR inhibition seem likely. In one, since the DNA sequences protected from DNase I digestion by SinR overlap the Spo0A binding sites, the two proteins could directly compete for binding, with one excluding the other. The data in Figures 2 and 6 show that the presence of each of the two proteins alters the ability of the other to modulate transcription, supporting the competition model. Our kinetic data demonstrate that Spo0A~P binding is rapidly reversible (20), so if competition between SinR and Spo0A~P occurs, it is likely to be a dynamic process.

The second mechanism arises from the observation that zinc induces DNase I HS sites at the *spoIIG* promoter. The position of these sites was compatible with known zinc-binding sequences (27). These HS sites are usually interpreted as reflecting DNA distortion and it has been shown that zinc binding kinks DNA (28,29). Binding of SinR to the *spoIIG* promoter induced DNase I HS sites at the same positions as did zinc, suggesting that SinR caused similar DNA deformations. The HS sites induced by SinR were near the 0A boxes required for Spo0A~P stimulation of *spoIIG* transcription, so that SinR could allow Spo0A~P binding but prevent its interaction with RNA polymerase by altering the conformation of the DNA. Since either of these mechanisms is dependent on SinR inhibiting activation of transcription by Spo0A~P, they can be extended to other Spo0A~P-dependent promoters, so that it is likely that SinR directly represses

transcription of all three stage II operon promoters, rather than exerting its effect via repression of the *spo0A* gene.

Since SinR forms stable complexes with DNA in the presence of zinc, we predict that SinR binding requires bent DNA. In many EMSAs, we found a SinR-dependent shift in the RNA polymerase–DNA complex that was more reproducible than formation of the SinR–DNA complex. We interpret this finding to indicate that RNA polymerase stabilizes SinR binding. This stabilization could be due to direct interactions between RNA polymerase and the SinR tetramers or because RNA polymerase alters the conformation of the DNA, in a manner analogous to zinc alteration of DNA structure.

We examined the SinR binding sites at the *spoIIG* promoter and the *lac* operator along with the known binding sites at the *spo0A*, *spoIIA* and *aprE* promoters for a consensus SinR binding site. We found that the only highly conserved sequence was TTGT. This sequence is common to the known recognition sites for several DNA binding proteins, including LacI, λ Cro and 434 repressor (30–32). However, there were no other common sequences among the SinR binding sites either upstream or downstream of the TTGT sequence. The importance of the TTGT sequence for SinR binding is supported by the work of Mandic-Mulec *et al.* (11), in which mutation of the G residue resulted in decreased binding of SinR to the *spo0A* promoter. The lack of an identifiable consensus binding sequence along with the data presented in this paper suggests that SinR binding may be dependent on the conformation of the DNA binding site rather than a specific recognition sequence.

ACKNOWLEDGEMENTS

This work was supported by a grant to G.B.S. from the National Science and Engineering Research Council of Canada. R.J.L. and J.A.B. were supported by the Wellcome Trust.

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