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Bacillus subtilis $\sigma^B$ is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase

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Communicated by Richard Losick, December 16, 1992 (received for review November 24, 1992)

ABSTRACT $\sigma^B$ is a secondary $\sigma$ factor of Bacillus subtilis. RNA polymerase containing $\sigma^B$ transcribes a subset of genes that are expressed after heat shock or the onset of the stationary phase of growth. Three genes (rsbV, rsbW, and rsbX), cotranscribed with the $\sigma^B$ structural gene (sigB), regulate $\sigma^B$-dependent gene expression. RsbW is the primary inhibitor of this system with the other genes acting upstream of RsbW in the $\sigma^B$ regulatory pathway. Evidence is now presented that RsbW inhibits $\sigma^B$-dependent transcription by binding to $\sigma^B$ and blocking the formation of a $\sigma^B$-containing RNA polymerase holoenzyme. Antibodies specific for either RsbW or $\sigma^B$ will coprecipitate both proteins from crude cell extracts. This is not due to the presence of both proteins on RNA polymerase. Western blot analysis of B. subtilis extracts that had been fractionated by gel-filtration chromatography revealed a single peak of RsbW that did not coelute with RNA polymerase and two peaks of $\sigma^B$ protein: one that eluted with RNA polymerase and a second that overlapped the fractions that contained RsbW. Reconstitution experiments were performed in which partially purified $\sigma^B$ and RsbW were added to core RNA polymerase and tested for their ability to influence the transcription of a $\sigma^B$-dependent promoter (ctc) in vitro. RsbW efficiently blocked $\sigma^B$-dependent transcription but only if it was incubated with $\sigma^B$ prior to the addition of the core enzyme.

$\sigma^B$ is a secondary $\sigma$ factor of Bacillus subtilis that is readily detected as a component of the holoenzyme population of vegetatively growing and stationary-phase cells (1–3). Although $\sigma^B$ was the first alternate $\sigma$ factor to be discovered in bacteria (2), its function remains elusive. Null mutations in the $\sigma^B$ structural gene confer no obvious phenotype on strains that carry them (4, 5). RNA polymerase containing $\sigma^B$ (E-$\sigma^B$) becomes active when B. subtilis enters stationary phase or is subjected to heat shock (refs. 6–10 and unpublished data). Under these conditions E-$\sigma^B$ activates both the synthesis of $\sigma^B$ itself and that of several additional genes that have been defined by their expression patterns and dependence in $\sigma^B$ (6, 8–10). The role of these target genes in B. subtilis physiology is unknown; however, if the conditions that trigger their expression are indicative of the types of signals that activate $\sigma^B$-dependent transcription in general, the $\sigma^B$ regulon would appear to participate in some aspect of stress response.

$\sigma^B$ is encoded by the third gene (sigB) of a four-gene operon that is primarily transcribed by the form of RNA polymerase holoenzyme that contains $\sigma^B$ itself (E-$\sigma^B$) (5, 11). The three other genes (rsbV, rsbW, and rsbX) encode regulatory proteins whose products have been shown, in genetic experiments, to control $\sigma^B$ synthesis and/or activity (6, 8, 11, 12). The sigB regulators appear to function in a pathway of negative control with RsbW as the primary inhibitor and RsbV as a modulator of RsbW’s repressive effects (6, 8). Null mutations in rsbW severely compromise B. subtilis growth (6, 8). This phenotype can be suppressed by null mutations in sigB itself. RsbX is also a negative regulator but its site of action is upstream of the RsbV/W pair in the control pathway (6, 8, 11). To our knowledge, the mode of RsbW inhibition has up to now been unknown. RsbW influences the expression of at least two $\sigma^B$-dependent promoters including the sigB operon itself (6, 8).

RsbV and RsbW are homologous to two other B. subtilis proteins called SpoI AA and SpoIIAB (11) that play a similar role in regulating the activity of the sporulation $\sigma$ factor $\sigma^F$ (13, 14). In the $\sigma^F$ system, the RsbW homologue (SpoIIAB) has been shown to inhibit transcription by E-$\sigma^F$ even if the $\sigma^F$ contains mutations that alter its promoter specificity (13, 14). This result indicates that SpoI AB controls E-$\sigma^F$ activity directly rather than the activity of the target promoters (13, 14). By analogy, RsbW could be playing a similar role in controlling the state of E-$\sigma^B$ within the cell. In this paper we provide evidence for the mechanism by which RsbW regulates $\sigma^B$-dependent transcription. Our results suggest that RsbW inhibits $\sigma^B$ activity by blocking the formation of the $\sigma^B$-containing holoenzyme. The data are consistent with RsbW functioning as an "anti-\sigma factor," binding to $\sigma^B$, and preventing its association with core RNA polymerase. Similar conclusions are drawn by Duncan and Losick (15) for the role of SpoI AAB in $\sigma^F$ regulation.

MATERIALS AND METHODS

B. subtilis Bacterial Strains. BSA105 (sigBPA28::PSPAC, pTET-I), BSA106 (sigBPA28::PSPAC, rsbV312, pTET-I), BSA107 (sigBPA28::PSPAC, rsbW313, pTET-I), and BSA116 (sigBPA28::PSPAC, sigB314, pTET-I) in which wild-type or mutant sigB operons are under the control of an isopropyl $\beta$-D-thiogalactopyranoside (IPTG)-inducible promoter have been described (7).

Immunoprecipitation of $\sigma^B$ and RsbW. Anti-$\sigma^B$, -RsbW, and -RsbV polyclonal and monoclonal antibodies were prepared by the University of Texas Health Science Center at San Antonio Hybridoma Facility from purified proteins overexpressed in Escherichia coli (7). Crude cell extracts of B. subtilis were incubated overnight with either anti-$\sigma^B$ or anti-RsbW antiserum at 4°C in binding buffer (16). Immune complexes were bound to protein-A-coupled Sepharose beads (Sigma), precipitated, washed, and fractionated by SDS/polyacrylamide gel electrophoresis (16). Western blot analyses were performed by standard means using antibodies specific for $\sigma^B$ and RsbW (17).

Reconstitution of RsbW-$\sigma^B$ Activities in Vitro. A 1.9-kbp DNA fragment, containing rsbV, rsbW, sigB, and a portion of rsbX (6), was cloned into an E. coli expression plasmid.

Abbreviations: E-$\sigma^B$, RNA polymerase containing $\sigma^B$; IPTG, isopropyl $\beta$-D-thiogalactopyranoside.

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(pRSET-Invitrogen, San Diego) and the proteins were over-expressed in *E. coli*. Crude cell extracts containing RsbV, RsbW, and σ^B_8 were fractionated by SDS/polyacrylamide gel electrophoresis. Gel slices containing the desired proteins were identified immunologically after the proteins had been eluted and renatured. B. subtilis core RNA polymerase was purified by standard procedures (3) from a B. subtilis strain (BSA80 (sigB314)) (6) that lacks σ^A_. For reconstitution studies, RsbW, σ^B, and core RNA polymerase were incubated at 4°C in renaturation buffer (18) prior to the addition of the DNA template and components of the transcription reaction mixture. The transcription reaction conditions and the analysis of the labeled RNA product have been described (2).

RESULTS

Immunoprecipitation of RsbW with σ^B_. RsbW inhibits σ^B-dependent transcription of at least two operons sigB and ctc (6, 8). Thus, either RsbW interacts independently with each promoter or it influences the activity of E-σ^B itself. The latter possibility is suggested by the observation that the RsbW homologue SpoIAB inhibits the ability of mutant forms of its target σ factor (σ^B) to transcribe promoters that are not normally regulated by SpoIAB (13, 14). If RsbW inhibits σ^B-dependent transcription by an effect on the transcribing enzyme rather than its target promoters, it is possible that RsbW exerts its influence by a direct protein–protein interaction with σ^B or E-σ^B.

To investigate possible interactions between RsbW and σ^B, polyclonal and monoclonal antibodies against components of the sigB operon were generated (7). Western blot analyses of the anti-σ^B and anti-RsbW polyclonal antibodies demonstrated that each of these reagents gives an appreciable response only to the antigen to which it was raised (Fig. 1). The absence of detectable cross-reactivity between the anti-σ^B and anti-RsbW antibodies for other B. subtilis proteins made them useful in coimmunoprecipitation studies to determine whether RsbW and σ^B can be found in an associated state. Crude cell extracts from strains synthesizing one or both of these proteins were incubated with either the anti-σ^B or anti-RsbW antibody. Immune complexes were precipitated with protein A-Sepharose, fractionated by SDS/polyacrylamide gel electrophoresis, and probed in Western blot analysis with anti-σ^B and anti-RsbW monoclonal antibodies (Fig. 2). Based on the Western blot data, the anti-σ^B antibody precipitated not only σ^B but also RsbW from the crude cell extract. This coprecipitation was specific for σ^B since the absence of either σ^B (lane 4) or anti-σ^B antibody (lane 3) from the precipitation mixture resulted in no detectable RsbW in the protein A-precipitated material. When the immunoprecipitated was repeated using anti-RsbW antiserum as the precipitating immunoglobulin, σ^B was coprecipitated. Based on the amount of antigen detected in the Western blot analysis, the anti-RsbW antibody was not as efficient in precipitating its target protein from the extract as was the anti-σ^B antibody in precipitating σ^B. However, even though the amount of material precipitated was less, σ^B was found to coprecipitate with RsbW (lane 6) by a process that depended on the presence of both RsbW (lane 8) and anti-RsbW antibody (lane 9). These experiments establish that σ^B and RsbW are part of a precipitable complex within the crude extract.

RsbV is a positive regulator of σ^B that appears to function by counteracting the ability of RsbW to inhibit σ^B. We therefore repeated the immunoprecipitation experiments using extracts prepared from B. subtilis mutant that lacked RsbV to ask whether its absence would influence the coimmunoprecipitation profiles or relative abundance of σ^B or RsbW in the precipitates. As can be seen in Fig. 2, the absence of RsbV had no effect on the immunoprecipitations regardless of whether anti-σ^B (lane 2) or anti-RsbW (lane 7) was the precipitating antibody. Apparently the potential antagonism exerted between RsbV and RsbW in vivo is not evident in our crude cell extracts.

σ^B Association with RsbW and RNA Polymerase. σ^B is normally isolated as a subunit of RNA polymerase. It is therefore possible that the coimmunoprecipitation of RsbW with σ^B represents binding of both of these proteins to RNA polymerase. To test this possibility, we fractionated a crude B. subtilis extract by gel filtration on Sephacryl S-200. Bacterial RNA polymerases are large molecules with molecular masses of ≈500 kDa. Molecules of this size are excluded from the gel matrix and exit the column in the lead fractions, whereas proteins not associated with RNA polymerase would be retained in the gel matrix. The column fractions were analyzed for σ^B-dependent RNA polymerase activity and the presence of the RsbW and σ^B proteins. Fig. 3 depicts our analysis. Fig. 3A is a Western blot of the fractions eluting from the gel filtration column using anti-σ^B and anti-RsbW as probes. There are two peaks of σ^B protein, one at fractions 2 and 3 and a second at fractions 6 and 7. RsbW is seen as a single broad peak that principally extends from fractions 6 to 9. Aside from σ^B and RsbW, the Western blot analysis detected two additional bands in lanes 8–10 that migrate slower than σ^B in our gel system. Their significance is unknown. Fig. 3B depicts an assay of the transcriptional activity of the fractions in which a DNA fragment containing a σ^B-dependent promoter (ctc) was used as a template (20). The template DNA was cleaved with a restriction endonuclease so that RNA polymerase initiating at the ctc promoter

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**Fig. 1.** Specificity of anti-σ^B and anti-RsbW polyclonal antisera. B. subtilis BSA105 (sigB, rsbW) (lanes 1 and 3), BSA116 (sigB314, rsbW) (lane 2), and BSA107 (sigB, rsbW313) (lane 4) were grown and induced with IPTG (1 mM) in Luria broth (19). Two hours after induction, cells were harvested and disrupted, and 100-μg protein samples were fractionated by SDS/PAGE (12.5% polyacrylamide gels), transferred electrophoretically to nitrocellulose, and probed with either anti-σ^B (lanes 1 and 2) or anti-RsbW (lanes 3 and 4) antisera. Bound antibody was visualized using alkaline phosphatase-conjugated goat immunoglobulin against mouse immunoglobulin (HyClone). The arrows indicate the positions of σ^B (B) and RsbW (w) on the Western blots.
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Fig. 2. Immunoprecipitation of B. subtilis extracts by anti-\(\sigma^B\) and anti-RsbW antibodies. Crude cell lysates were prepared from B. subtilis strains grown in Luria broth after 1 hr with 1 mM IPTG. The extracts were immunoprecipitated with either anti-\(\sigma^B\) antibody (lanes 1, 2, and 4) or anti-RsbW antibody (lanes 6–8), incubated without antibody (lanes 3 and 9), or ethanol-precipitated (lane 5). Samples were then fractionated by SDS/PAGE (12.5% polyacrylamide gels). The separated protein bands were transferred electro-phoretically to nitrocellulose and probed with a mixture of anti-\(\sigma^B\) and anti-RsbW monoclonal antibodies. Bound antibody was visualized as in Fig. 1. Lanes: 1, 3, 5, 6, and 9, BSA105 (sigB, rsbW); 2 and 7, BSA106 (sigB, rsbW, rsbV312); 4, BSA116 (sigB314, rsbW); 8, BSA107 (sigB, rsbV313). The arrows indicate the positions of the \(\sigma^B\) and RsbW proteins in the Western blots. The prominent bands at the center of the Western blots are the precipitating mouse antibodies that are bound by the alkaline phosphatase-conjugated secondary antibodies.

would generate a run-off transcript of 365 bases. The formation of the transcription of the transcript was analyzed by gel electrophoresis. Based on the abundance of the RNA product, the bulk of the E–\(\sigma^B\) activity was in fractions 2 and 3 with lesser amounts tralling into fractions 4 and 5. The pattern of E–\(\sigma^B\) activity paralleled total RNA polymerase activity, which, based on the level of transcription of poly (dA-dT), was predominantly in fractions 2 and 3 (data not shown). Fractions 2–5 contained approximately half of the \(\sigma^B\) that was detected in the Western blot analysis (Fig. 4A, lanes 2–5). The remainder of the \(\sigma^B\) was retained on the column and included in fractions that also contained RsbW (Fig. 4A, lanes 6 and 7). Unlike \(\sigma^B\), RsbW was not found to elute in the RNA polymerase-containing fractions. Based on the elution profiles of RNA polymerase, RsbW, and \(\sigma^B\), RsbW does not appear to be an RNA polymerase binding protein but may be a \(\sigma^B\) binding protein. The coincidental elution of a portion of the \(\sigma^B\) and RsbW proteins in the same fractions of the gel-filtration column and the immunoprecipitation result suggest that these proteins either directly interact or are part of an undefined complex that does not include RNA polymerase.

**RsbW Inhibits \(\sigma^B\)-Dependent Transcription in Vitro.** The finding that \(\sigma^B\) can be fractionated by gel filtration into populations that either coelute with RNA polymerase or RsbW suggests that \(\sigma^B\) can associate with both of these proteins but that the associations are mutually exclusive. We attempted to examine this hypothesis by reconstructing RsbW-dependent inhibition of \(\sigma^B\) in vitro. The sigB operon products RsBV, RsbW, and \(\sigma^B\) were expressed to high levels in E. coli as a transcriptional fusion of the sigB operon to a T7 RNA polymerase-dependent expression system (pRSET). The E. coli extract containing these proteins was fractionated by SDS/polyacrylamide gel electrophoresis. The gel was sequentially sliced and the proteins contained in these slices were eluted, renatured, and analyzed immunologically for \(\sigma^B\), RsbW, and RsBV. Fig. 4A depicts the analysis for these proteins in a series of eight consecutive slices from the gel. Samples were spotted onto nitrocellulose and probed with anti-\(\sigma^B\), anti-RsbW, or anti-RsBV monoclonal antibodies.

\(\sigma^B\), RsbW, and RsBV proteins were most abundant in slices 3, 6, and 8, respectively. Each of the proteins present in these fractions was relatively free of the other proteins for which we probed. We used fraction 3 as a source of \(\sigma^B\) and examined the effect of the RsbW containing fractions to inhibit \(\sigma^B\) activity. The assay system was similar to that used in Fig. 3 to determine the location of E–\(\sigma^B\) after gel filtration. The “run off” ctc transcript is seen when a sample of fraction 3 protein is added to purified core RNA polymerase (Fig. 4B, lane 2) but absent if the transcribing enzyme is core RNA


Fig. 3. Fractionation of \(\sigma^B\) and RsbW by gel filtration of crude B. subtilis extracts. Cells of BSA105 (Δ28:reporter sigB, rsbW, sigB, rsbV, rsbX) were grown in Luria broth and harvested after 1 hr with 1 mM IPTG. Approximately 3 × 10^11 cells were resuspended in 4 ml of 10 mM Tris, pH 8.0/50 mM NaCl/10 mM EDTA/1 mM MgCl_2/5% (vol/vol) phenylmethylsulfonyl fluoride/3 mM dithiothreitol and disrupted by passage through a French pressure cell at 15,000 psi (1 psi = 6.9 kPa). Debris was removed by centrifugation (18,000 × g for 15 min), and 1.5 ml of the supernatant was loaded onto a 50-ml column of Sephacyrl S-200 (Pharmacia), which was developed using the resuspension buffer. After elution of the void volume, 1.5-ml fractions were collected and analyzed for \(\sigma^B\) and RsbW proteins (A) or E–\(\sigma^B\) activity (B). (A) Samples (200 μl) of the indicated fractions were precipitated with 2 vol of ethanol and analyzed for \(\sigma^B\) and RsbW proteins by Western blot as in Fig. 1. Lane C is 25 μl of the unfraccionated extract precipitated and processed along with the fractionated samples. The positions of \(\sigma^B\) and RsbW (w) are indicated. (B) Samples (10 μl) of the indicated fractions were added to a 100-μl in vitro transcription reaction mixture in which the template DNA [a 950-bp DNA fragment encoding the promoter-proximal portion of the ctc gene cloned into pBR322 (20, 21)] was cut at a site 365 bp downstream of the \(\sigma^B\)-recognized promoter. In vitro-synthesized 32P-labeled RNA was subjected to electrophoresis through a 6% polyacrylamide gel containing 7 M urea. After electrophoresis, the polyacrylamide gel was exposed to x-ray film to visualize the bands. The arrow indicates the position of the expected \(\sigma^B\)-dependent transcript.
Fig. 4A, lane 6, an amount of RsbW that was sufficient to block detectable ctc transcript transcription can only partially inhibit this transcription if some E-σB is allowed to form prior to its addition. We conclude that the primary target for RsbW is free σB and that E-σB is relatively resistant to RsbW activity.

**DISCUSSION**

We (6) and others (8) had previously shown that RsbW is the primary inhibitor of σB-dependent transcription at two distinct operons. We have now presented evidence that the mechanism involved in this inhibition involves a direct interplay between RsbW and σB. RsbW and σB can be coimmunoprecipitated from crude B. subtilis extracts by using antibodies directed against either RsbW or σB. Since fractionation of B. subtilis extracts separates the σB component between RNA polymerase-containing and RsbW-containing fractions, RNA polymerase does not appear to be involved as a part of the RsbW and σB complex. The partitioning of σB between RNA polymerase and RsbW strongly suggests that the association of σB with each of these proteins is mutually exclusive. The hypothesis that RsbW interacts with σB, but not E-σB, is strengthened by the in vitro reconstitution experiment in which the order of addition of the reaction components significantly affects the outcome of the transcription reaction. RsbW is maximally effective in inhibiting σB-dependent transcription when incubated with σB prior to the addition of core RNA polymerase. Incubation of σB with core RNA polymerase prior to adding RsbW restrains RsbW's ability to block transcription although it still partially inhibits this reaction. This partial inhibition could reflect an interaction between RsbW and σB that is not yet bound to RNA polymerase at the time of RsbW addition or that becomes released from the enzyme during a round of the transcription cycle and prevented from participating in subsequent rounds of transcription. Regardless of the basis of the inhibition, however, the data clearly implicate free σB and not E-σB as the preferred substrate for RsbW inhibition in vitro.

A simple model for RsbW inhibition that is consistent with our current data would have RsbW form a physical complex with σB thereby preventing σB from joining to core RNA polymerase to generate an active holoenzyme. Although it is possible that RsbW blocks σB activity by a chemical modification of σB rather than by sequestering it in a complex, we consider this unlikely. This inhibition could reflect an interaction between RsbW and σB that blocked σB's ability to function as a σ factor (Fig. 4) was carried out in a buffer solution (renaturation buffer) with no obvious donor molecule for a chemical modification (18). If RsbW is modifying σB, it cannot be doing so by the more conventional means of modification that occur in other regulatory systems (i.e., phosphorylation, methylation, etc.). In addition, the RsbW and σB coding regions within the sigB operon overlap (11) thereby translationally coupling the synthesis of σB to the synthesis of RsbW (7). Such a device would favor the appearance of these proteins in an equal molar ratio. An equivalent ratio of synthesis between RsbW and σB would be anticipated for proteins that participate in complex formation rather than those that share an enzyme-substrate relationship.

If RsbW is blocking σB activity by joining to it and masking its RNA polymerase binding potential, how is this association regulated? Although control of transcription factor activity by protein–protein interactions is relatively rare in prokaryotes, it has been described in yeast and higher eukaryotic systems. The *Saccharomyces cerevisiae* Gal4 protein, a transcriptional activator of galactose pathway genes, is inhibited when bound to the Gal80 protein and released from this inhibition by the activity of a second protein, Gal3 (22). In higher eukaryotes, the E2F transcription factor is activated or repressed by binding to other cellular proteins.
particular, its activity is inhibited by binding to the retinoblastoma gene product and released from this inhibition by the adenovirus E1A gene product or cell cycle proteins (23, 24). In these cases, release of the transcriptional activator is accomplished by an interaction with a third protein that disrupts the complex. RsbV is a candidate for such a protein in the RsbW-\(\sigma^B\) system, since it has been shown genetically to counter the negative control of RsbW (6, 8). We would assume that RsbV could interact with the \(\sigma^B\)-RsbW complex to release \(\sigma^B\). Preliminary experiments support a direct interaction between RsbV and RsbW-\(\sigma^B\). By using an anti-RsbV monoclonal antibody to probe the proteins that are precipitated from crude B. subtilis extracts by anti-\(\sigma^B\) antibody, we observed RsbV as part of the \(\sigma^B\)-RsbW complex; however, at this point, we have no evidence of whether its presence in the complex is due to a direct association with \(\sigma^B\) or RsbW.

There are at least two environmental conditions that can activate \(\sigma^B\): the onset of stationary phase (6, 8) and heat shock (unpublished data). \(\sigma^B\) activation upon entry into stationary phase requires RsbV (6–8). We suggest that stationary phase generates a signal that activates RsbV to release \(\sigma^B\) from RsbW. Heat-shock activation does not require RsbV (unpublished data). Either the RsbW-\(\sigma^B\) complex is thermolabile or RsbW is dissociated from \(\sigma^B\) by a second release factor yet to be determined. If this second possibility is correct, then RsbW could be a common target for several distinct release factors that respond to different environmental cues. A similar model of multiple effectors has been proposed for the E2F protein and its inhibition by the retinoblastoma protein (24).

The existence of a \(\sigma\) factor binding protein that inhibits its activity is not unique to \(\sigma^B\). Duncan and Losick (13) describe a similar regulatory circuit for the control of \(\sigma^B\), and Ohnishi et al. (25) provided evidence for a protein inhibitor of the Salmonella typhimurium flagellum-specific \(\sigma\) factor. Anti-\(\sigma\) factor proteins may be common devices for controlling the activity of secondary \(\sigma\) factors. Unraveling how they are controlled to modulate the activity of specific \(\sigma\) factors will now require a detailed genetic and biochemical analysis.

We thank R. Losick for communicating data prior to publication and D. Kolodrubetz for a critical reading of the manuscript. This work was supported by National Science Foundation Grant DMB-8916793 and National Institutes of Health Grant IR35GM48220-01. A.K.B. was a Tom Slick Fellow of the Southwest Foundation for Biomedical Research.