Evidence that a Single Monomer of Spx Can Productively Interact with RNA Polymerase in Bacillus subtilis

Ann A. Lin and Peter Zuber

Division of Environmental & Biomolecular Systems, Institute of Environmental Health, Oregon Health & Science University, Beaverton, Oregon, USA

Spx activates transcription initiation in Bacillus subtilis by directly interacting with the C-terminal domain of the RNA polymerase (RNAP) holoenzyme α subunit, which generates a complex that recognizes the promoter regions of genes within the Spx regulon. Many Gram-positive species possess multiple paralogs of Spx, suggesting that two paralogous forms of Spx could simultaneously contact RNAP. The composition of Spx/RNAP was examined in vitro using an Spx variant (SpxΔCHA) bearing a 12-amino-acid deletion of the C terminus (SpxΔC) and a hemagglutinin (HA) epitope tag and Spxc-Myc, a full-length Spx with a C-terminal myelocytomatosis oncoprotein (c-Myc) epitope tag. All Spx/RNAP complexes bearing deletion or C-terminal-tagged variants were transcriptionally active in vivo and in vitro. Reaction mixtures containing SpxΔCHA and Spxc-Myc combined with RNAP were applied to either anti-HA or anti-c-Myc affinity columns. Eluted fractions contained RNAP with only one of the epitope-tagged Spx derivatives. The resin-bound RNAP complex bearing a single epitope-tagged Spx derivative was transcriptionally active. In vivo production of SpxΔC and SpxΔCHA followed by anti-HA affinity column chromatography of a cleared lysate resulted in retrieval of RNAP with only the SpxΔCHA derivative. Binding reactions that combined active Spxc-Myc, inactive Spx(R60E)ΔCHA, and RNAP, when applied to the anti-HA affinity column, yielded only inactive Spx(R60E)ΔCHA/RNAP complexes. The results strongly argue for a model in which a single Spx monomer engages RNAP to generate an active transcriptional complex.

Throughout phylogeny, positive transcriptional control plays an important role in cellular decision-making (45). The mechanisms that activate transcription initiation in response to environmental and metabolic signal inputs are varied and involve complex sensory functions linked to specific macromolecular interactions conducted at targeted chromosomal loci. For many regulatory systems, these interactions involve contacts between transcriptional regulators and RNA polymerase (RNAP). In prokaryotes, RNA polymerase, composed of β, β’, α, 2 α, and σ subunits, contains multiple target surfaces that can engage positive regulatory proteins, which function to direct RNAP to the specific regulatory regions of genes that are under their control (17). The σ subunit, bearing two domains that occupy the protein’s N and C termini (NTD and CTD, respectively), is a common target for regulatory protein interaction (18). A classic example is the class I and II positive control exerted by the cyclic AMP (cAMP) receptor protein-cAMP complex (CRP-cAMP), which targets specific regulatory regions of genes that are under its control (19, 20). Encounters with any one of a variety of toxic agents can result in an elevated Spx concentration and Spx activation (1, 5, 8, 21). Encounters with any one of a variety of toxic agents can result in an elevated Spx concentration and Spx activation (1, 5, 8, 36, 46, 49).

Spx protein bears a CXXC disulfide redox center that controls its transcription-stimulating activity (34). Spx in its oxidized, disulfide form productively interacts with RNAP, forming a complex that contacts promoters bearing a cis-acting element (having the sequence a/ɔGCA followed by an AT-rich element (having the sequence a/ɔGCA followed by an AT-rich sequence)

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Address correspondence to Peter Zuber, pzuber@ebs.ogi.edu.
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sequence) located immediately upstream of the promoter −35 region (31, 44). While Spx cannot by itself interact with DNA, a complex consisting of Spx and αCTD interacts with DNA that bears the cis-acting element of the Spx-controlled promoter (31). From mutational analysis of promoter DNA and studies of Spx/αCTD-DNA interactions, evidence was obtained suggesting that two Spx/αCTD complexes might participate in Spx-controlled promoter recognition (31). The notion of two Spx proteins, each binding an α subunit, engaging RNAP has significant implications when considering the roles of multiple Spx paralogs that are encoded within the genomes of several low-GC-content Gram-positive species (19, 43, 47), including pathogenic streptococci. Could two paralogueous forms of Spx engage RNAP, thus expanding the promoter recognition and sensory capabilities of the Spx/RNAP complex?

In the study reported here, the composition of the Spx/RNAP complex was examined using differentially affinity-tagged Spx proteins. Results of affinity interaction chromatography experiments performed with in vitro-assembled complexes of epitope-tagged Spx and RNAP, as well as complexes collected from extracts of cells expressing tagged Spx derivatives, indicate that the Spx/RNAP complex bears a single Spx monomer.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains and plasmids are listed in Table S1 in the supplemental material. The Bacillus subtilis strains used in this study are derivatives of H642 and were grown at 37°C in 2× yeast extract-tryptone (2×YT) or Difco sporulation medium (DSM) (14). Escherichia coli DH5α was used for plasmid construction and was grown at 37°C in 2×YT liquid or on Luria-Bertani (LB) solid medium containing 1.2% agar (Difco). For overproduction and purification of Spx proteins in E. coli, ER2566 strains (New England Biolabs) bearing spx-overexpressing plasmids were grown at 37°C in LB liquid medium. Antibiotic concentrations used were as previously reported (6).

Construction of epitope-tagged Spx derivatives. To construct the expression plasmids producing epitope hemagglutinin (HA)- or c-Myc-tagged Spx proteins, DNA fragments encoding HA and c-Myc tags were generated by annealing the forward and reverse oligonucleotides whose sequences correspond to HA or c-Myc codons (α292/α30.3 for c-Myc tag; αAL35/αAL34 for HA tag [oligonucleotide sequences are listed in Table S2 in the supplemental material]), followed by PCR. The fragments were cleaved with BamHI and SalI and inserted into pUC18 that was cleaved with BamHI and SalI to create pAL39 and pAL40 carrying the αCTD-DNA interactions, evidence was obtained suggesting that two Spx variants by double crossover, spx with upstream and downstream flanking regions was PCR amplified with oligonucleotides αML02-07/αMMN07-357 and αAL43/αAL44, respectively, and the resultant PCR products were cloned into pUC19 to create pAL78 and pAL79. For antibiotic selection, a kanamycin-resistant (Km) cassette isolated from pDG783 (12, 13) was inserted immediately downstream of the spx sequence in pAL78 to create pAL80. To create a clone containing αCTD for integration into the spx locus, a downstream sequence was cut from pAL79 with BamHI and EcoRI and inserted into pAL80 to create pAL81, which was used for B. subtilis transformation. For ectopic, IPTG-inducible spx-DHA expression from the amylE locus, pAL45 (mentioned above) was used to transform B. subtilis.

β-Galactosidase assays. Strains bearing a trxB-lacZ fusion were grown at 37°C overnight on DSM agar plates supplemented with appropriate antibiotics. The overnight cultures were used to inoculate the same liquid medium at a starting optical density at 600 nm (OD600) of 0.02. When the OD600 of the cultures reached 0.4, the cultures were divided into two flasks and 1 mM IPTG was added to one of the flasks. Samples were collected every 30 min, and β-galactosidase activity was assayed as previously described (30); data are presented as Miller units (27).

Protein purification. His-tagged, αs-depleted RNAP (Sδδ-RNAP) was purified from αs mutant B. subtilis strain ORB5833 [p[pc-His6o sigA�L364)], in which the Leu366 substitution in αs weakens the interaction with the RNAP core enzyme (52). B. subtilis cells were grown in 2×YT liquid containing chloramphenicol and neomycin at 37°C until the OD600 of the culture reached 0.8 to 0.9 and were then harvested by centrifugation at 2,000 × g. The pellets were frozen at −80°C prior to purification. Nickel-nitriotrifluoracetate (Ni-NTA; PerfectProxy™ 5′) affinity columns, heparin column, and Bio-Rad High Q column chromatography was performed as previously described (31, 44). (Note that Spx was previously reported to interact with Ni-NTA resin [32], but the resin used in this study did not bind native Spx protein.) RNAP was purified and stored at −20°C in buffer containing 10 mM Tris-HCl (pH 7.8), 100 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, and 50% glycerol.

The genes specifying αs and Spx variants were cloned in plasmid pTYB4 (Terminus Impact-CN system; New England Biolabs). The products of the recombinant plasmids bear a self-cleavable intein domain and a chitin-binding domain positioned at the C termini. αs was overproduced from plasmid pSN64 (28) in E. coli ER2566 and purified by using chitin resins (New England Biolabs) followed by a Bio-Rad High Q column. Purified protein was dialyzed and stored at −80°C in buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM MgCl2, and 10% glycerol. Spx variants were expressed from the pTYB4 derivatives listed in Table S1 in the supplemental material. As previously described, Spx proteins were purified by using a chitin column followed by a Bio-Rad High Q column (29). For the affinity interaction assay, all of the Spx proteins were concentrated to 10 μM and stored at −80°C in buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM KCl, and 5% glycerol.
In vitro transcription. A linear trxB promoter DNA template was generated by PCR with oligonucleotides oDYR07-32 and oDYR07-52 (30) to yield a fragment that would direct the synthesis of a 66-nucleotide (nt) transcript. For the reaction, the template (10 nM) and RNAP (25 nM), together with 25 nM α, were incubated without or with 75 nM Spx protein in 17.8 μM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, and bovine serum albumin (BSA; 50 mg/ml). After a 10-min incubation at 37°C, 2.2 μl of a nucleotide mixture (200 mM ATP, GTP, and CTP, 10 mM UTP, 5 mM [α-32P]UTP) was added. After incubation at 37°C for 8 min, 10 μl of stop solution (1 M ammonium acetate, 0.1 mg of yeast RNA, and 0.03 M EDTA) was added to the reaction. The mixture was precipitated with ethanol, and the pellet was dissolved with 5 ml of formamide dye (0.3% xylene cyanol, 0.3% bromophenol blue, and 12 mM EDTA dissolved in formamide). The samples were heated at 90°C for 2 min and were applied to an 8% polyacrylamide–urea gel. The dried gels were scanned on a Typhoon Trio+ variable imager (GE Healthcare).

To confirm that pulldown complexes were active, the RNAP/Spx complex-bound anti–HA resin was directly used in transcription reactions. An in vitro anti-HA affinity interaction assay was performed on a reaction mixture containing 0.25 μM SAd-RNAP, 0.25 μM α, and 2.5 μM SpxΔCHA. After washing, instead of elution of the protein with triethylamine, resin was suspended in 40 μl of reaction buffer (RB; 10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 5 mM MgCl₂). A 10-μl volume of resin suspension was directly added in the transcription reaction buffer with 10 nM trxB (−115 to approximately +47) promoter DNA, and the reaction was performed as outlined above.

For the in vitro transcription with RNAP/Spx/DNA complex-bound resins, an in vitro anti-HA affinity interaction assay was performed with a reaction mixture containing 0.25 μM SAd-RNAP, 0.25 μM α, and 2.5 μM SpxΔCHA, and 0.2 μl trxB (−50 to approximately +20) promoter DNA in the presence of 1 mM rATP. A 10-μl volume of resin suspension, or 1 μl of input or flowthrough from the column, was added to separate reaction tubes and the in vitro transcription was performed as described above.

In vitro affinity interaction assay. To detect proteins interacting with HA-tagged Spx in vitro, 40 μl of the anti-HA affinity matrix was pre-equilibrated with 10 column volumes (CV) of RB and blocked with 4 CV of blocking buffer (5% skim milk in RB) followed by washing with 8 CV of RB. For the reaction, 0.25 μM His-tagged SAd-RNAP and 2.5 μM SpxΔCHA were incubated with or without 0.25 μM α in 150 μl of RB at room temperature. After 20 min, the protein mixture was then applied to the anti-HA affinity column followed by washing with 10 CV of washing buffer (0.05% Tween 20 in RB) and with 10 CV of RB. The protein complex was eluted with 0.25 μl of 100 mM triethylamine (pH 11.5) and neutralized with 1/10 volume of RB containing 0.25 μM α. The composition of the protein complex was analyzed on the 15% sodium acrylamide gel electrophoresis (PAGE) and by Western blotting using anti-HA antibody.

To determine whether Spx proteins form homodimers in solution, gel filtration chromatography was performed. Purified Spx or SpxΔCHA was applied to Bio-Gel P-60 polyacrylamide gel (Bio-Rad) and run with buffer containing 10 mM Tris-HCl (pH 6.8), 100 mM KCl, and 1% glycerol. The void volume and the calibration curve were determined by using a gel filtration LMW calibration kit (GE Healthcare) and plotting a standard Kₘ-logMW graph. The partition coefficient, Kₛ, of each protein was calculated by using the equation Kₛ = (Ve - Vs)/(Ve - Vo), where Ve = void volume, Vs = elution volume, and Vo = geometric column volume. By correlating the Kₛ value of Spx protein to the calibration curve, the composition of Spx protein in the solution was determined.

RESULTS

Epitope-tagged versions of Spx are active in vivo and in vitro. Allelic variants of spx were constructed that specified modifications to the C-terminal ends of each product. Oligonucleotide PCR primers specifying the HA and c-Myc epitopes were used to create alleles encoding C-terminal epitope-tagged ver-
sions of Spx and a deletion variant missing the 12 C-terminal amino acid residues. Previous studies had shown that the deletion of the 12 C-terminal residues created an active, protease-resistant version of Spx (SpxΔC [see below]; C. M. Chan and P. Zuber, unpublished data). The epitope-tagged variants included HA-tagged SpxΔC (SpxΔCHA), as well as c-Myc-tagged, full-length Spx protein (Spxc-Myc) (see Fig. S1A in the supplemental material). SpxΔCHA and Spxc-Myc were tested in vivo by ectopic expression from an IPTG-inducible promoter in the amyE locus (Fig. 1A and B), expression of which is stimulated by Spx (44). A construct bearing an allele of spx encoding the protease-resistant form of Spx (SpxF37D [35]) was used in parallel with SpxΔCHA in reactions using holo-RNAP (see Fig. S2A and B in the supplemental material). The result suggested that SpxΔCHA and Spxc-Myc interact with RNAP confirmed by affinity chromatography. To examine the composition of Spx/RNAP, affinity chromatography designed to detect Spx bound to RNAP was undertaken. RNAP and SpxΔCHA were combined in a 1:10 molar ratio in a binding reaction that was applied to an anti-HA affinity column that had been blocked with buffer containing milk to prevent nonspecific protein binding. Elution of SpxΔCHA with associated RNAP was accomplished with a high pH buffer and confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). RNAP did not interact with the anti-HA column (Fig. 2B). Spxc-Myc bound to RNAP could also be captured by an anti-c-Myc affinity column, and eluted fractions could be resolved by SDS-PAGE, showing Spxc-Myc and bound RNAP (Fig. 2D), while RNAP showed weak interaction with the anti-c-Myc column. By the use of the sigA(L366A) B. subtilis mutant (kindly provided by C. P. Moran, Jr., Emory University), a Δa defect in RNAP core enzyme interaction (52) in a preparation of RNAP depleted of Δa (SAd-RNAP) was obtained (see Fig. S1A in the supplemental material). As shown in Fig. S1B in the supplemental material, RNAP from the sigA(L366A) mutant did not transcribe the trxB template, but transcripts were detected after purified Δa protein was applied to the reaction. Further addition of Spx stimulated transcription from the Spx-controlled trxB promoter. Binding of Δa depleted RNAP with SpxΔCHA was observed by anti-HA affinity chromatography, although binding affinity of SpxΔCHA with RNAP was higher when Δa was present (Fig. 2A and C). Repeat binding reaction mixtures containing SAd-RNAP and SpxΔCHA were applied to the anti-HA Affi-Gel column. SAd-RNAP coeluted with SpxΔCHA; however, the amount of bound enzyme was less than that obtained in reactions using holo-RNAP (see Fig. S2A and B in the supplemental material). The result suggested that Δa is required for optimal Spx-RNAP interaction. Note that RNAP holoenzyme and SAd-RNAP showed no affinity for the anti-HA Affi-Gel column (see Fig. S3 in the supplemental material).

To test the interaction of RNAP with Spx mutants that show defects in transcriptional activation, anti-HA Affi-Gel interaction reactions were assembled with proteins having substitutions at residue positions known to be required for Spx activity. An SpxΔCHA variant bearing a G52R mutation, shown previously to disrupt Spx-αCTD interactions, was combined with RNAP holoenzyme or SAd-RNAP. After incubation, the mixture was applied to the anti-HA affinity column. Immobilized complexes were eluted at high pH and analyzed by SDS-PAGE (Fig. 3A and B). The Spx-RNAP interaction was quantified by reduced activity of the two epitope-tagged Spx variants was due to the sensitivity of the proteins to YjbH-mediated proteolysis catalyzed by ClpXP.

To confirm that the SpxΔCHA and Spxc-Myc proteins were active, the proteins were produced as intein-chitin binding domain fusions and purified by chitin column affinity chromatography and intein cleavage (see Fig. S1A in the supplemental material). After further purification by anion-exchange chromatography, each protein was applied to a transcription reaction mixture containing trxB promoter DNA and purified RNAP holoenzyme (see Fig. S1B in the supplemental material). Both epitope-tagged versions of Spx stimulated trxB transcription in vitro, using the same transcriptional start site as the wild-type Spx/RNAP complex.

**SpxΔCHA and Spxc-Myc interaction with RNAP confirmed by affinity chromatography.** To examine the composition of Spx/RNAP, affinity chromatography designed to capture Spx bound to RNAP was undertaken. RNAP and SpxΔCHA were combined in a 1:10 molar ratio in a binding reaction that was applied to an anti-HA affinity column that had been blocked with buffer containing milk to prevent nonspecific protein binding. Elution of SpxΔCHA with associated RNAP was accomplished with a high pH buffer and confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). RNAP did not interact with the anti-HA column (Fig. 2B). Spxc-Myc bound to RNAP could also be captured by an anti-c-Myc affinity column, and eluted fractions could be resolved by SDS-PAGE, showing Spxc-Myc and bound RNAP (Fig. 2D), while RNAP showed weak interaction with the anti-c-Myc column. By the use of the sigA(L366A) B. subtilis mutant (kindly provided by C. P. Moran, Jr., Emory University), a Δa defect in RNAP core enzyme interaction (52) in a preparation of RNAP depleted of Δa (SAd-RNAP) was obtained (see Fig. S1A in the supplemental material). As shown in Fig. S1B in the supplemental material, RNAP from the sigA(L366A) mutant did not transcribe the trxB template, but transcripts were detected after purified Δa protein was applied to the reaction. Further addition of Spx stimulated transcription from the Spx-controlled trxB promoter. Binding of Δa depleted RNAP with SpxΔCHA was observed by anti-HA affinity chromatography, although binding affinity of SpxΔCHA with RNAP was higher when Δa was present (Fig. 2A and C). Repeat binding reaction mixtures containing SAd-RNAP and SpxΔCHA were applied to the anti-HA Affi-Gel column. SAd-RNAP coeluted with SpxΔCHA; however, the amount of bound enzyme was less than that obtained in reactions using holo-RNAP (see Fig. S2A and B in the supplemental material). The result suggested that Δa is required for optimal Spx-RNAP interaction. Note that RNAP holoenzyme and SAd-RNAP showed no affinity for the anti-HA Affi-Gel column (see Fig. S3 in the supplemental material).
The Spx\(\Delta\)CHA mutant showed significantly reduced affinity for RNAP holoenzyme, and the data were in line with previous results (31, 37). A similar result was obtained for reaction mixtures containing a mutant form of RNAP holoenzyme bearing a subunit with a Y263C substitution that does not interact efficiently with Spx (see Fig. S4 in the supplemental material) (33). These experiments confirmed that spx and rpoA mutations that compromise Spx-RNAP interaction disrupt Spx-RNAP complex formation. In contrast, the Spx(R60E) and Spx(C10A) mutants were expected to interact with RNAP. The Spx(R60E)\(\Delta\)CHA and Spx(C10A)\(\Delta\)CHA mutant proteins interacted with RNAP holoenzyme with an affinity only slightly less than that of the parent Spx\(\Delta\)CHA but showed noticeably reduced affinity for SAd-RNAP (Fig. 3C and D). Spx(R60E) and Spx(C10A) exert negative transcriptional control in vivo by direct interaction with RNAP but are severely defective in transcriptional activation, which is the result of poor binding to their DNA target when complexed with CTD (31, 34, 50).

Spx\(\Delta\)CHA and Spxc-Myc compete for RNAP interaction. One view of the composition of Spx/RNAP is that two Spx proteins, one bound to each subunit, would associate with RNAP and generate a transcription activation complex in which two cis-acting Spx response elements would be utilized to stimulate transcription from an Spx-controlled promoter (31). Experiments were conducted with epitope-tagged Spx variants to determine if Spx/RNAP contains two Spx monomers. A reaction was assembled in which Spx\(\Delta\)CHA and Spxc-Myc were combined with holo-RNAP, and then applied to the anti-HA Affi-Gel affinity column. As shown in Fig. S1 in the supplemental material, the two Spx variants can be distinguished by SDS-PAGE according to the differences in their molecular weights. Elution of the complex from the anti-HA column should have resulted in coelution of RNAP and Spxc-Myc protein if the transcription complex contained two Spx monomers. The gel profile of the eluted fraction showed that only the HA-tagged Spx variant and associated holo-RNAP (Fig. 4A and B) or SAd-RNAP (Fig. 4C and D) bound to the anti-HA column, while Spxc-Myc was found in the flowthrough and wash fractions. The elution was carried out at high pH, and the fraction collected was not active in an in vitro transcription reaction. However, the immobilized complex on the anti-HA Affi-Gel was active in transcription reactions when combined with trxB promoter DNA and radiolabeled nucleotide mixture (see Fig. S5 in the supplemental material). The results indicate that the active Spx/RNAP complex is composed of a single Spx protein. The same result was obtained when the reaction was applied to an anti-c-Myc Affi-Gel (see Fig. S6 in the supplemental material). Following elution and gel electrophoresis, Spxc-Myc was found to be associated with RNAP, and a protein of lower molecular weight was barely detectable beneath the Spxc-Myc...
band, which might have reflected a small amount of RNAP nonspecifically bound to the column (see Fig. S6 in the supplemental material) or a degradation product of Spxc-Myc, which we observed in some of the SDS-PAGE and Western blot analyses.

The experiment was repeated by first purifying RNAP in the presence of Spx/H9004 CHA and Spxc-Myc protein by the use of an Ni-NTA column, taking advantage of the rpoC allele encoding a His-tagged 6 subunit (40). Figure 5 shows the input of RNAP (IR) followed by Spx/H9004 CHA and Spxc-Myc input (IS). Elution with imidazole recovers RNAP bound to the Spx proteins. This was then applied to the Affi-Gel anti-HA column, and the eluted fraction again contained only Spx/H9004 CHA and RNAP, while Spxc-Myc was found in the flowthrough fraction.

The possibility that promoter DNA was required for the interaction of two Spx monomers with the Spx-activated transcription complex was tested using the two epitope-tagged forms of Spx. A segment of trxB promoter DNA used in previous electrophoretic mobility shift assay (EMSA) studies containing a sequence from −50 to +20 (31) was added to the binding reaction mixture containing Spx/H9004 CHA, Spxc-Myc, and RNAP. The reaction mixture was applied to the anti-HA column, and the eluted fraction again contained only SpxΔCHA and RNAP, while Spxc-Myc was found in the flowthrough fraction.

The finding that only one Spx protein establishes contact with RNAP suggests that other forms of Spx, such as the reported paralogous forms in certain Gram-positive bacteria, could potentially compete for RNAP. The Spx-RNAP pulldown experiment using SpxΔCHA and Spxc-Myc proteins was repeated with a 3-fold-higher concentration of Spxc-Myc. As predicted, when excess Spxc-Myc was present in the binding reaction that was applied to the anti-HA Affi-Gel column, less RNAP coeluted with Spx/H9004 CHA (Fig. 4A and B). To rule out the possibility that the extended C terminus of the Spxc-Myc protein inhibited interaction of a second Spx monomer with Spx/RNAP, wild-type Spx protein was added to the binding reaction mixture containing Spx/H9004 CHA and Spxc-Myc. The two Spx proteins are nearly the same molecular weight and cannot be distinguished by SDS-PAGE. However, the same reduction in Spx/H9004 CHA-RNAP interaction was observed when a 3-fold excess of Spx was added to the binding reactions (data not shown). Competition for SAd-RNAP was also observed when Spxc-Myc was added in excess to the binding reaction mixture containing SpxΔCHA and SAd-RNAP (Fig. 4C and D).

Evidence that Spx is a monomer in solution. A possible explanation for why two differentially tagged versions of Spx do not engage RNAP simultaneously might be that Spx forms a dimer in solution and that the dimer of SpxΔCHA is in contact with the two 6 subunits of RNAP. Previous reports argue against this possibility. The crystal structure of an in vivo-assembled Spx/αCTD complex shows no interaction between Spx monomers (20), and no Spx in dimeric form was generated in an Spx/αCTD complex assembled in vitro (37). Second, ArsC, the structural homolog of Spx, functions as a monomer (24). We performed a glutaraldehyde cross-linking experiment (see Fig. S7 in the supplemental material),

FIG 3 Effect of Spx mutants on RNAP interaction in vitro. An interaction assay was carried out with anti-HA affinity chromatography. Interaction between Spx mutants and RNAP holo (A) or SAd-RNAP (C) was analyzed by SDS-PAGE, and the intensity of each subunit of RNAP holo (B) or SAd-RNAP (D) was quantified and is presented as a ratio to the intensity of SpxΔCHA. Abbreviations: WT, SpxΔCHA; G52R, Spx(G52R)ΔCHA; C10A, Spx(C10A)ΔCHA; R60E, Spx(R60E)ΔCHA; I, input; E, elution. Only input and elution results are shown.
using Spx αCTD and α protein alone as positive controls, indicating that the majority of SpxCHA and wild-type Spx protein in solution is monomeric whereas cross-linked versions of Spx/αCTD and α/α complexes can be detected. Notably, the Spx/αCTD cross-linked product is the size that would be predicted if one monomer of Spx interacted with αCTD. Size exclusion chromatography with molecular weight markers showed that SpxCHA elutes from the column between MW 13 and 29, indicating a monomeric form of the protein (see Fig. S7B in the supplemental material).

FIG 4 In vitro RNAP binding competition between SpxΔCHA and Spxc-Myc. SpxΔCHA and an equal or 3-fold-molar-excess amount of Spxc-Myc were incubated with RNAP holo-RNAP (A) or SAd-RNAP (C) in binding reactions. The complex was captured with an anti-HA column, and the composition of the Spx/RNAP complex was analyzed by SDS-PAGE and Western blotting using antibodies against Spx. (B and D) The relative amount of RNAP subunit binding to SpxΔCHA in the presence of competitor Spxc-Myc was quantified and normalized against the reaction mixtures containing no Spxc-Myc (for which the ratio of RNAP subunit to Spx was given the value 1.0) and is presented as an RNAP subunit/Spx ratio.

FIG 5 In vitro two-column interaction assay of Spx/RNAP complex. His-tagged RNAP holo or SAd-RNAP was immobilized on an Ni-NTA column, and a mixture of SpxΔCHA and Spxc-Myc at a 1:1 molar ratio was applied to the RNAP prebound Ni column. The Spx/RNAP complex was eluted with buffer containing 200 mM imidazole, and the elution fraction was directly applied to an anti-HA affinity column. The Spx/RNAP complex was captured through the SpxΔCHA interaction and eluted at high pH. All the fractions from the two columns were collected and analyzed by SDS-PAGE and Western blotting using Spx antibodies. Abbreviations: Iᵢ, RNAP input; FTᵢ, RNAP flowthrough; Iₛ, Spx input; FTₛ, Spx flowthrough; W, wash; E/Iᵢ, elution from Ni column and input for anti-HA column; FT, complex flowthrough; E, elution.
Wild-type Spx does not confer activity to an Spx(R60E)CHA/ RNAP complex. Evidence from affinity interaction experiments indicates that a single Spx protein interacts with RNAP to form the transcription activation complex, although it was possible that a small, undetectable amount of RNAP bound to two Spx proteins was captured on the anti-HA column. The possibility that two Spx proteins are capable of binding RNAP but that only one is active was tested by the following experiment. A binding reaction was assembled that contained Spxc-Myc, RNAP, and Spx(R60E)CHA and was applied to the anti-HA Affi-Gel column. Affi-Gel beads were retrieved to determine the transcriptional activity of the complex. As was observed in the previous experiments, only the HA-tagged form of Spx was detected in the elution fraction, as determined by SDS-PAGE and Western blot analyses (Fig. 7A). The anti-HA beads containing immobilized Spx(R60E)CHA-RNAP complex had no activity when trxB promoter DNA was added to immobilized mutant Spx/RNAP complexes on anti-HA resin in the presence of NTPs and radiolabeled UTP (Fig. 7B). No transcriptional activity was detected in the resin-bound Spx/RNAP complex when trxB promoter DNA was added to the binding reaction with RNAP, Spxc-Myc, and Spx(R60E)CHA prior to column chromatography (Fig. 7C). These results indicate that Spx does not productively associate with an RNAP complex bearing an inactive Spx variant.

A single Spx monomer interacts with RNAP in vivo. The data of Fig. 2 to 6 were generated in experiments using purified Spx and RNAP to assemble complexes in vitro. To gain an understanding of Spx/RNAP complex composition in vivo, an experiment was conducted to recover RNAP from cells in which Spx variants with differentially modified C termini were produced. Two spx alleles were expressed; one resided in the spx locus and encoded SpxCHA, which is active but missing the C-terminal 12 amino acids, while at the amyE locus, a version of spx encoding SpxpCHA was expressed from an IPTG-inducible promoter. SpxCHA could be distinguished from the SpxpCHA protein on SDS-PAGE by its higher molecular weight. The strain bearing the two spx alleles also contained an allele of rpoC (β') whose product has a C-terminal His tag designed for RNAP purification (39).

Cells of the spx diploid strain were grown in DSM to an OD600 of 0.4 and then treated with IPTG. Cells were harvested after 30 min and lysed by French press. The lysate was applied to an anti-HA column, which was washed and finally treated with high-pH elution buffer. Western analysis of the column fractions showed that only the SpxCHA protein was eluted from the column (Fig. 8A), while the ΔC product was found in the flowthrough fraction. This result supported previous structural data and the experimental data in Fig. S7 in the supplemental material, indicating that Spx does not form a homomultimer (30). Western analysis of the fractions using an anti-His tag showed that RNAP copurified with SpxCHA in the affinity interaction assay reaction.

A sample of lysate, prepared in the same manner as described above, was applied to an Ni-chelate chromatography column. SDS-PAGE of the eluted RNAP showed that the two Spx products coeluted (Fig. 8B, showing replicate results), indicating that both are capable of interacting with RNAP in vivo. The eluted fraction was then applied to the anti-HA Affi-Gel column, which was washed and then treated with high-pH elution buffer. The fractions were applied to two SDS-PAGE gels, and the resolved proteins were blotted onto nylon filters for Western analysis using anti-Spx and anti-His tag antibody. The RNAP and SpxCHA were detected in the eluted fraction of the Affi-Gel anti-HA column. SpxpCHA, which coeluted with RNAP from the Ni-chelate column, was observed in the flowthrough fraction but was not detectable in the eluted fraction. The results show that both in vivo-produced Spx derivatives were able to interact with RNA polymerase but that only one Spx protein was observed within an Spx/RNAP complex.

DISCUSSION

Previous studies have shown that Spx contacts RNAP αCTD to form a complex that can interact with a cis-acting element of Spx-controlled promoters (31, 37). The report of these studies posed a hypothesis that two Spx monomers could contact RNAP, forming a complex that could recognize promoters bearing either one or two Spx-responsive sequence elements. Such Spx-responsive elements have been found in the trxA, trxB, nfrA, and ytpQ genes, all of which are transcriptionally activated by Spx. The sequence resides immediately upstream of the −35 region of the core promoter in all four promoters, while in the trxB and trxA genes, a second element was thought to be located in a sequence overlapping with the −35 element (31). Several scenarios could be envisioned to explain the mechanism of Spx/RNAP-promoter interaction. Two Spx monomers, each occupying a binding surface on
the RNAP α subunit, might be required for optimal activation, or two Spx monomers bind to RNAP but only one functions in stimulating transcription. A third possibility is that a single Spx monomer engages RNAP and generates the DNA-binding Spx/αCTD complex for promoter recognition. The evidence reported here strongly supports the third scenario: a single Spx monomer interacts with RNAP holoenzyme to form the active Spx-stimulated transcription complex.

In this third scenario, one of the two α subunits, α bound to β or αII to β', is the target of Spx interaction, and binding to either one could result in formation of a transcriptionally active complex. Alternatively, Spx must bind to only one of the two α subunits to productively engage RNAP. In this case, interaction between Spx and RNAP might involve multiple contacts, not only with αI or αII, but with another subunit, as binding to one or the other would position Spx to contact another specific interaction surface on one of the RNAP subunits. In the Affi-Gel experiments, more RNAP coeluted with Spx when holoenzyme was present in the binding reactions than when SAd-RNAP was applied to the reaction, suggesting that σ^A enhances Spx-RNAP interaction and that the σ subunit might contribute an interaction surface. However, the exact basis of this effect remains to be investigated.

An excess of Spx protein was applied to each RNAP binding reaction, and yet a significant amount of RNAP remained unbound to Spx. This might have been due, in part, to the aforementioned preference of Spx for σ^A-bearing RNAP holoenzyme but could also have been due to the blocking conditions used to prevent nonspecific binding of protein to the Affi-Gel column. Adding σ^A in 500-fold excess did not significantly increase the portion of RNAP that could bind Spx. Recently reported proteomic analysis provided evidence that approximately 60% of holoenzyme RNAP is bound to σ^A (7). While the RNAP preparation used in our studies appeared to be quite pure after a 3-column purification, we cannot rule out the presence of RNAP-associated proteins, such as alternative sigma subunits, that might weaken Spx-RNAP interaction. At present, we do not know if Spx can engage alternative holoenzyme forms or if Spx can associate with elongating RNAP.

CRP-cAMP is capable of interacting with either α subunit as part of its mechanism for stimulating transcription initiation (4). In class I transcriptional activation, the target promoter bears a catabolite activator protein (CAP) site centered at position −61 and is followed by an AT-rich sequence upstream of the −35 core promoter element. It is estimated that 3 in 4 class I activated complexes involve contact between a CRP dimer and α subunit (16, 17), whereas the CAP site is not essential for Spx-stimulated transcription (4).

FIG 7 Binding reactions of RNAP with Spxc-Myc and mutant Spx(R60E)CHA do not yield active Spx/RNAP complex bound to anti-HA column. (A) Anti-HA Affi-Gel interaction assay conducted in the presence of trxB promoter DNA. RNAP was incubated with SpxΔCHA or Spx(R60E)CHA in the absence or presence of trxB (−50 to +20)DNA, and the protein mixture was applied to an anti-HA Affi-Gel column. After washing and elution, the complex was analyzed by SDS-PAGE and Western blotting with Spx antibodies. For in vitro transcription by using complex-bound beads, the beads were resuspended with reaction buffer and directly added into the transcription reaction. (B) Spx/RNAP-complex-bound beads were added into a transcription mixture containing a trxB long (−118 to +47) or short (−50 to +20) promoter template. (C) Spx/RNAP/trxB-complex-bound beads were added to an in vitro transcription reaction. The trxB transcripts are marked by arrows. I, input; FT, flowthrough; E, elution; B, complex-bound anti-HA beads.
The established target of Spx being the RNAP CTD complex. In light of the evidence presented here, as well as interactions between RNAP and CRP-cAMP and its class I promoter (16, 22). As with CRP, a single Spx might interact with αCTD and form a productive complex with RNAP. The result of our experiments also suggests that not only αCTD but also other RNAP subunits, such as the β subunit, may be involved in the interaction with Spx.

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