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# Transcription activation by phage Φ29 protein p4 is mediated by interaction with the α subunit of *Bacillus subtilis* RNA polymerase

(protein–DNA interactions/protein–protein interactions/gene expression)

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**ABSTRACT** **Regulatory protein p4 from *Bacillus subtilis* phage φ29 activates transcription from the viral late A3 promoter by stabilizing $\sigma^A$-RNA polymerase at the promoter as a closed complex. Activation requires an interaction between protein p4 and RNA polymerase mediated by the protein p4 carboxyl-end, mainly through residue Arg-120. We have obtained derivatives of *B. subtilis* RNA polymerase α subunit with serial deletions at the carboxyl-end and reconstituted RNA polymerase holoenzymes harboring the mutant α subunits. Protein p4 promoted the binding of purified *B. subtilis* RNA polymerase α subunit to the A3 promoter in a cooperative way. Binding was abolished by deletion of the last 15 amino acids of the α subunit. Reconstituted RNA polymerases with deletions of 15 to 59 residues at the α subunit carboxyl-end could recognize and transcribe viral promoters not activated by protein p4, but they had lost their ability to recognize the A3 promoter in the presence of protein p4. In addition, these mutant reconstituted RNA polymerases could not interact with protein p4. We conclude that protein p4 activation of the viral A3 promoter requires an interaction between the carboxyl-end of protein p4 and the carboxyl-end of the α subunit of *B. subtilis* RNA polymerase that stabilizes the RNA polymerase at the promoter.**

Transcription of *Bacillus subtilis* phage φ29 genome is regulated by the product of the viral gene 4, protein p4. This regulator activates the promoter for late genes, A3 (1, 2), and simultaneously represses promoters A2b and A2c, responsible for the expression of the main early genes (3, 4). Protein p4 binds at the A3 promoter to a site located between positions −58 and −104 with respect to the transcription start point (5) and activates transcription by stabilizing the binding of RNA polymerase (RNAP) to the promoter as a closed complex, having little effect on the rest of the steps of the initiation process (6). The activation requires a direct interaction between protein p4 and *B. subtilis* $\sigma^A$-RNA polymerase through the protein p4 carboxyl-end, mainly through residue Arg-120 (6–8). Substitution of residue Arg-120 by glutamine, alanine, or lysine leads to a p4 derivative that can bind to DNA efficiently but is unable to activate transcription; it can neither stabilize the RNAP at the A3 promoter nor contact the RNAP (7, 8).

Eubacterial RNAP holoenzyme is a multicomponent enzyme composed of at least five subunits, $\alpha_2\beta\beta'\sigma$. The sigma factor determines the promoter specificity of RNAP and also interacts with certain transcriptional activators (9–14). The elongation of the transcript is undertaken by the core enzyme ($\alpha_2\beta\beta'$). The β subunit has a catalytic function, the β′ subunit has nonspecific DNA binding properties, and the α subunit plays several roles (reviewed in refs. 15–17). The α subunit is a dimer in solution ($\alpha_2$) and has two independent domains connected by a flexible interdomain linker (18, 19). The N-terminal domain contains determinants for α–α dimerization, α–β interaction, and, possibly, α–β′ interaction (20–22); it serves as the initiator for the RNAP assembly. The C-terminal domain of the *Escherichia coli* α subunit acts as a receiver region interacting with several transcriptional regulators in a way that leads to transcription activation (reviewed in ref. 16). This domain, which is capable of dimerization, can specifically recognize a supplementary A+T-rich promoter element, named UP element, present upstream of the −35 region of certain promoters (18, 23–25).

We have found that protein p4 can induce the binding of purified *B. subtilis* α subunit to the late A3 promoter and that this depends on an interaction held between the protein p4 carboxyl-end and the C-terminal region of the α subunit. The use of reconstituted RNAPs with nested deletions at the α subunit carboxyl-end has indicated that the last 15 residues of RNAP α subunit are required for protein p4-dependent transcription activation. The implications of these results for the transcription activation mechanism of protein p4 are discussed.

## METHODS

**Cloning and Construction of Deletion Mutants of *B. subtilis rpoA* Gene, Coding for RNAP α Subunit.** Plasmid pT7Bα was obtained by assembling into the pT7-7 expression vector (26) two DNA fragments from plasmids pSA136 and pSW102 (provided by Chester Price, University of California), containing the 5′- and 3′-segments of *B. subtilis rpoA* gene (27) as follows. A *Nde*I–*Eco*RI fragment from pSA136 containing the 5′-half of *rpoA* gene was ligated between the *Nde*I and *Eco*RI sites of plasmid pT7-7, generating plasmid pT7BNα. The *Eco*RI–*Bsi*WI fragment from plasmid pSW102 (the *Bsi*WI end was filled in with Klenow enzyme and dNTPs), containing the 3′-half of the *rpoA* gene, was cloned between the *Eco*RI and *Sma*I sites of plasmid pT7BNα. The resulting plasmid, pT7Bα, was transformed into *E. coli* BL21(DE3) cells.

Deletion mutants at the 3′-end of the *rpoA* gene were obtained by PCR-site-directed mutagenesis. One of the primers hybridized upstream of the *Eco*RI site in the *rpoA* gene, while the mutagenic primers (phosphorylated) were designed to introduce stop codons at desired positions of the *rpoA* gene. The DNA fragments obtained were cut with *Eco*RI and cloned into plasmid pT7BNα between the *Eco*RI and *Sma*I sites, using the *E. coli* strain BL21(DE3) as host.

**Overproduction and Purification of the RNAP α Subunit.** Overexpression of the *rpoA* gene from plasmid pT7Bα was performed as described (28). Cells were lysed, washed with

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Abbreviation: RNAP, RNA polymerase.
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buffer A [50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 7 mM 2-mercaptoethanol, and 5% (vol/vol) glycerol], and supplemented with 1 M NaCl, and the cell debris was eliminated by centrifugation at 30,000 × *g*. Polyethylenimine was added to the supernatant [final concentration 0.25% (vol/vol)], and the aggregated material was eliminated by centrifugation at 12,000 × *g*. The supernatant was diluted to 0.35 M NaCl, which caused aggregation of the overexpressed $\alpha$ subunit, which was collected by centrifugation at 12,000 × *g*. The pellet was resuspended in buffer A supplemented with 1 M NaCl; proteins were precipitated with 65% ammonium sulfate and resuspended in 100 ml of buffer A. The sample was passed through a Whatman P11 phosphocellulose column and loaded onto a Whatman DEAE-cellulose column, which was eluted with buffer A containing 150 mM NaCl. Fractions containing the overexpressed $\alpha$ subunit were diluted to 50 mM NaCl and loaded onto an heparin-agarose column. The $\alpha$ subunit was eluted with 30% ammonium sulfate, precipitated with 65% ammonium sulfate, and resuspended and dialyzed in storage buffer [buffer A; 50% (vol/vol) glycerol and 0.2 M KCl]. The $\alpha$ subunit obtained was >95% pure. No contamination by *E. coli* RNAP $\alpha$ subunit was detected.

Overexpression of *rpo*A mutants was carried out in a similar way. Mutant $\alpha\Delta56$ was purified as the wild-type, except that the protein eluted from the DEAE-cellulose column at 125 mM NaCl, and the heparin-agarose column was substituted by a fast protein liquid chromatography mono-Q column and eluted with a 0–0.5 M NaCl gradient in buffer A. Mutants $\alpha\Delta15$ and $\alpha\Delta37$ were found in the pellet of the 30,000 × *g* centrifugation after cell lysis. The pellets were solubilized in buffer A with 6 M guanidinium hydrochloride and dialyzed against buffer A containing 0.2 M NaCl. Samples were centrifuged, diluted to 50 mM NaCl, loaded onto a DEAE-cellulose column and eluted with buffer A containing 125 mM NaCl. Fractions containing the mutant $\alpha$ subunits were diluted, loaded onto a fast protein liquid chromatography mono-Q column and eluted as mutant $\alpha\Delta56$. Fractions containing the mutant $\alpha$ subunits were concentrated with Centricon-Amicon 30 and dialyzed against storage buffer. Protein concentration was determined by the method of Lowry *et al.* (29) and by laser scanning densitometry of Coomassie blue-stained polyacrylamide gels with adequate standards. The purity of the mutant proteins was similar to that of the wild-type $\alpha$ subunit.

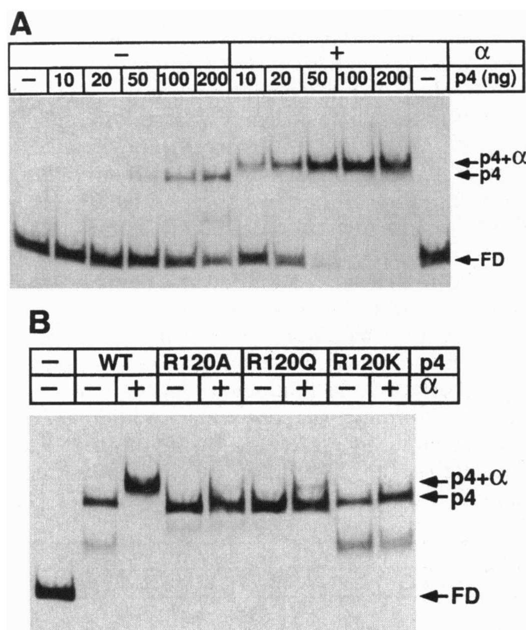


FIG. 1. Binding of the RNAP $\alpha$ subunit to the A3 promoter. (*A*) Cooperativity in the binding of protein p4 and the purified RNAP $\alpha$ subunit to the A3 promoter. An end-labeled 198-bp DNA fragment containing the complete A3 promoter (core promoter plus protein p4 binding site) was incubated with increasing concentrations of protein p4 (0.01 to 0.2 $\mu$g, 16 to 320 nM) in the absence or presence of the $\alpha$ subunit (2 $\mu$g, 1.16 $\mu$M); the complexes formed were resolved in a band-shift gel. (*B*) Role of the protein p4 activating surface in the stabilization of purified $\alpha$ subunit at the A3 promoter. The end-labeled DNA fragment indicated above was incubated in the absence or presence of the purified RNAP $\alpha$ subunit (1.16 $\mu$M) and in the presence of either wild-type protein p4 or p4 mutant derivatives R120A, R120Q, or R120K (0.83 $\mu$M each). The complexes formed were resolved in a band-shift gel. The extra band migrating between free DNA and the p4–DNA complex corresponds to nonspecific binding of protein p4 to the DNA fragment (7, 8). FD, free DNA; p4, protein p4–DNA complex; and p4+$\alpha$, protein p4-$\alpha$ subunit–DNA complex.

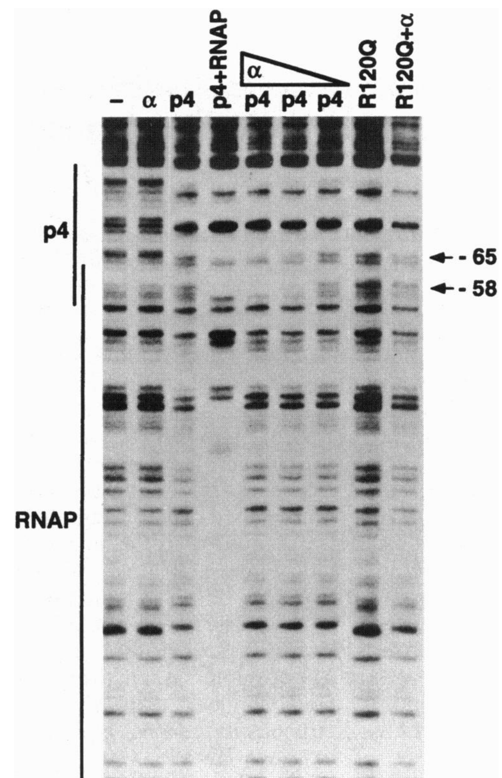


FIG. 2. DNase I footprinting of the binding of protein p4 and purified $\alpha$ subunit to the A3 promoter. An end-labeled 237-bp DNA fragment containing the A3 promoter was incubated with either $\alpha$ subunit (0.29 to 1.16 $\mu$M), protein p4 (0.83 $\mu$M), protein p4 mutant R120Q (0.83 $\mu$M), RNAP holoenzyme (36 nM), or the indicated combinations of these proteins, and the complexes were analyzed by DNase I footprinting. The binding sites for protein p4 and RNAP are indicated. In the presence of protein p4, but not in its absence, the purified $\alpha$ subunit protects from DNase I cleavage positions −58 and −65 relative to the transcription start site (indicated with arrows).

*B. subtilis* $\sigma^A$ subunit was purified as described (30). *B. subtilis* RNA polymerase holoenzyme and protein p4 were purified as described (1, 7).

**Reconstitution of *B. subtilis* RNA Polymerase.** To substitute the wild-type $\alpha$ subunit by the purified mutant derivatives in RNA polymerase, wild-type holoenzyme (400 $\mu$g) was denatured in 2 ml of buffer D [50 mM Tris·HCl (pH 8), 20% (vol/vol) glycerol, 10 mM $MgCl_2$, 10 $\mu$M $ZnCl_2$, 1 mM EDTA, 10 mM DTT, and 6 M guanidine hydrochloride] and mixed with a 20-fold molar excess (1600 $\mu$g) of purified $\alpha$ subunit (either wild-type or mutant). Purified $\sigma^A$ subunit (100 $\mu$g) was also included, giving a final protein concentration of 1.05 mg/ml. The holoenzyme was renatured by overnight dialysis against 500 ml of reconstitution buffer [50 mM Tris·HCl (pH 8), 0.2 M KCl, 20% (vol/vol) glycerol, 10 mM $MgCl_2$, 10 $\mu$M

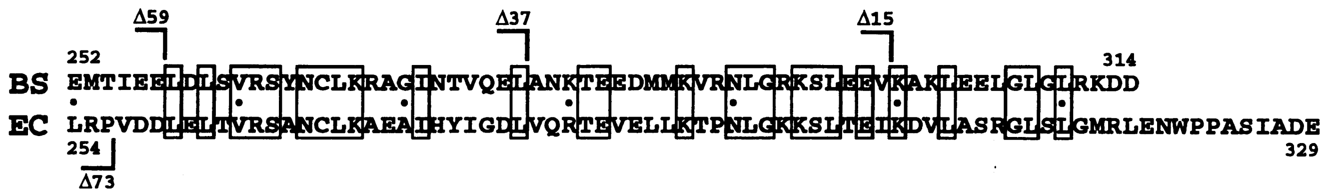

FIG. 3. Deletion mutants obtained at the carboxyl-end of the RNAP α subunit. The sequence of the last residues of the C-terminal domain of *B. subtilis* (residues 250–314) and *E. coli* (residues 254–329) α subunits are shown. The deletions reported in this work for *B. subtilis* α subunit (last 15 residues, Δ15; last 37 residues, Δ37; and last 59 residues, Δ59), and the smallest deletion reported for *E. coli* α subunit (last 73 residues, Δ73; see ref. 16), are indicated. The residues that are identical in both polypeptides are boxed.

$ZnCl_2$, 1 mM EDTA, and 1 mM DTT), diluted with 2 ml of the same buffer without glycerol, and concentrated with the Centricon-Amicon 30. The holoenzyme was separated from the nonreconstituted subunits by centrifugation (270,000 × *g*; 24 h) through a 10-ml glycerol gradient [15–30% (vol/vol)] in reconstitution buffer. Fractions containing the holoenzyme were pooled and concentrated as above. $\sigma^A$ subunit was added in a 10-fold molar excess; samples were incubated for 1 h at 30°C, concentrated, and dialyzed against storage buffer. RNAPs reconstituted with the mutant α subunits contained <10% of wild-type α subunit. The specific activity of reconstituted RNAPs was measured through their ability to transcribe from the phage ϕ29 promoters not activated by protein p4. The specific activities obtained, expressed as percent relative to that of the nonreconstituted RNAP, were as follows: wild-type RNAP, 21%; αΔ15-RNAP, 43%; αΔ37-RNAP, 12%; and αΔ59-RNAP, 7%. All assays with the reconstituted RNAPs were performed using equivalent amounts of the holoenzymes in terms of specific activity.

**Band-Shift Assays.** Binding reactions contained, in 20 μl, end-labeled DNA (see below), 25 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 90 mM ammonium sulfate, 2 μg of poly[d(I-C)] and, where indicated, protein p4, purified *B. subtilis* α subunit, or $\sigma^A$-RNAP at the concentrations indicated in the figure legends. After incubation for 10 min at 37°C, the complexes formed were analyzed in a 4% polyacrylamide gel at 4°C. Band-shift assays with purified α subunits were performed with a 198-bp *Nde*I DNA fragment, obtained from plasmid pFRC56 (31), which contains the entire A3 promoter and the protein p4 binding site at its center. The assays with reconstituted RNAPs were performed with a 237-bp DNA fragment containing the entire A3 promoter, obtained from plasmid pFRC64 (3) with *Hin*dIII and *Kpn*I. The 89-bp *Eco*RI–*Taq*I DNA fragment containing protein p4 binding site but lacking the RNAP binding sequences at the A3 promoter, was obtained from plasmid pFRC64. In this case, ammonium sulfate was omitted from the binding reaction. All DNAs were labeled by filling in the 3′-recessive ends with Klenow enzyme in the presence of [α-$^{32}$P]dATP, dTTP, dCTP, and dGTP.

**DNase I Footprinting.** Reaction conditions were as for band-shift assays, except that ammonium sulfate was not included. Proteins were added as specified in the figure legends. The 237-bp *Hin*dIII–*Kpn*I DNA fragment from plasmid pFRC64, labeled at the *Hin*dIII end, was used. DNase I treatment was as described (7).

***In Vitro* Transcription Assays.** Reactions contained, in 25 μl, 25 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM NaCl, 1 mM DTT, 45 mM ammonium sulfate, 200 μM of each UTP, CTP, and GTP, 80 μM [α-$^{32}$P]ATP (2 μCi; 1 Ci= 37 Gbq), 2 μg of poly[d(I-C)], 2 μg of bovine serum albumin, and 9 nM template DNA. The templates used were a 344-bp DNA fragment containing the A3 promoter, obtained by PCR amplification from plasmid pFRC64 (3), and a 260-bp DNA fragment containing the phage ϕ29 C2 promoter, obtained from viral DNA. The concentration of nonreconstituted RNAP was 9 nM; reconstituted RNAPs were added in amounts sufficient to obtain a transcription activity from the C2 promoter equivalent to that obtained with nonreconstituted RNAP. Protein p4 was used at 0.8 μM. Reactions proceeded at 37°C for 15 min and were stopped and processed as described (6). Transcripts were resolved in denaturing polyacrylamide gels, detected by autoradiography, and quantified by laser scanning densitometry. As an internal control, all reactions carried a DNA fragment with the C2 promoter, that is independent of protein p4. The transcription signals obtained from the A3 promoter were normalized in all cases with respect to those obtained from the C2 promoter.

## RESULTS AND DISCUSSION

**Protein p4 Induces the Binding of the α Subunit of *B. subtilis* RNAP to the A3 Promoter.** As stated above, activation of the A3 promoter requires a direct interaction between protein p4 and $\sigma^A$-RNA polymerase, which stabilizes the polymerase at the promoter as a closed complex (6). The transcriptional activators analyzed to date that interact with RNAP have been found to do so through the RNAP subunits α or σ. Those activators binding at or upstream from position −60 relative to the transcription start site normally interact with the α subunit (16). Protein p4 binding site is centered at position −82, which suggests that it might interact with the α subunit of RNAP. To test this possibility, we purified the α subunit of *B. subtilis* RNAP and assayed by gel retardation if protein p4 could facilitate its binding to the A3 promoter. The results, shown in Fig. 1*A*, indicate that the α subunit cannot bind by itself to the A3 promoter, but, in the presence of protein p4, it generates a new band with a mobility slower than that of protein p4, suggesting that both protein p4 and the α subunit are bound to DNA. Furthermore, the binding of protein p4 and the α subunit to the promoter was cooperative: addition of α subunit to a sample containing protein p4 in amounts that retard only a small fraction of the DNA shifted all the DNA to the position corresponding to the p4-α subunit–DNA complex (Fig. 1*A*).

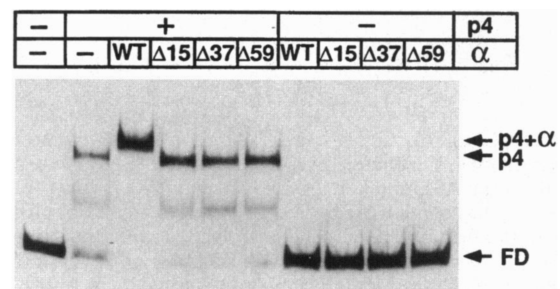


FIG. 4. Binding of the mutant α subunits lacking the last 15, 37, or 59 residues to the A3 promoter in the absence or presence of protein p4. A 198-bp end-labeled DNA fragment containing the A3 promoter was incubated with either protein p4 (0.83 μM), wild-type α subunit (1.16 μM, indicated as WT), or the different α subunit deletion derivatives (Δ15, Δ37, or Δ59, 1.16 μM each), and the complexes formed were resolved in a band-shift gel. The complexes detected in the presence of protein p4 (indicated as p4), or of protein p4 and the α subunit (denoted as p4+α), are indicated with arrows. FD, free DNA.

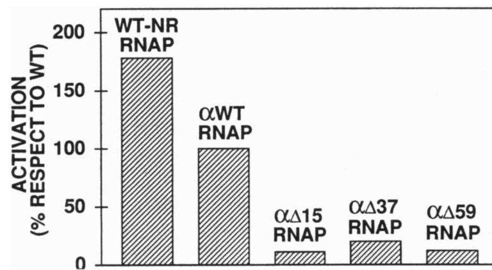


FIG. 5. Activity of RNAP holoenzymes reconstituted with mutant $\alpha$ subunits. The ability of protein p4 to activate transcription from the A3 promoter in the presence of RNAPs reconstituted with either wild-type or mutant $\alpha$ subunits was measured by *in vitro* transcription; the behavior of nonreconstituted RNAP is shown for comparison. DNA template (9 nM) and RNAP (9 nM for nonreconstituted RNAP, and equivalent amounts in terms of specific activity for reconstituted RNAPs) were incubated in the absence or presence of protein p4, and the transcripts originating at the A3 promoter were analyzed by denaturing PAGE. The graphic shows the activation ratio obtained expressed as percent relative to that obtained for the RNAP reconstituted with the wild-type $\alpha$ subunit. The specific activity of reconstituted RNAPs was calculated measuring their ability to transcribe from the phage $\phi$29 promoters that do not require activation by protein p4.

Addition of an unrelated protein such as bovine serum albumin instead of the $\alpha$ subunit did not help protein p4 to bind to the promoter (data not shown), ruling out the possibility that the $\alpha$ subunit was just increasing the effective concentration of protein p4. Interestingly, binding of the $\alpha$ subunit was not detected when, instead of the wild-type protein p4, its mutant derivatives R120A, R120Q, or R120 K were used (Fig. 1*B*). These mutant proteins are known to bind to DNA correctly but are unable to interact with RNAP, and therefore they can neither stabilize the RNAP at the promoter nor activate transcription (7, 8). The mutation is thought to affect to the most critical residue of protein p4 activation surface. Therefore, these results suggest that protein p4 bound at the A3 promoter interacts through residue Arg-120 with the $\alpha$ subunit of RNAP, promoting its binding to the promoter. In agreement with this hypothesis, protein p4 did not promote the binding of purified $\sigma^A$ subunit to the promoter (data not shown).

DNase I footprints confirmed the above results and showed that, in the presence of protein p4, the $\alpha$ subunit modifies the

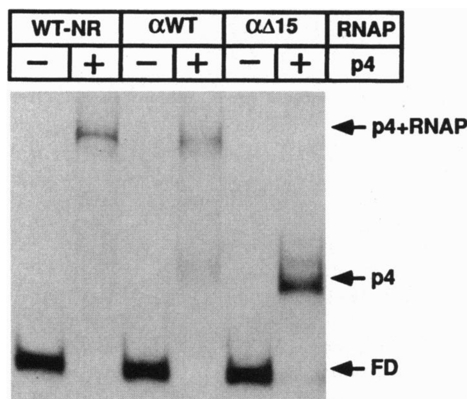


FIG. 6. Protein p4-mediated stabilization of the binding of reconstituted RNAPs to the A3 promoter. An end-labeled 237-bp DNA fragment containing the A3 promoter was incubated, in the absence or presence of protein p4 (830 nM), with nonreconstituted RNAP (indicated as WT-NR), with RNAP reconstituted either with the wild-type $\alpha$ subunit (indicated as $\alpha$WT), or with the $\alpha$ mutant lacking the last 15 residues of the C-end (indicated as $\alpha\Delta15$). Equivalent amounts of RNAP were used in all cases in terms of specific activity. The complexes formed were resolved in a band-shift gel. FD, free DNA; p4, protein p4–DNA complex; and p4+RNAP, protein p4-RNAP–DNA complex.

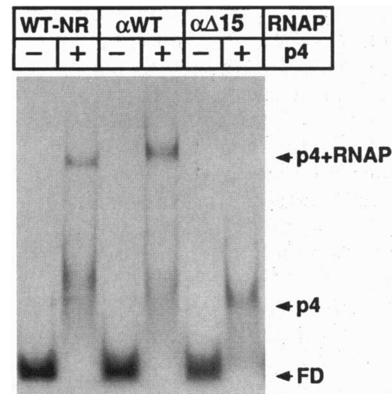


FIG. 7. Interaction between protein p4 and RNAP. An end-labeled 89-bp DNA fragment containing the binding site for protein p4, but not that for RNAP, was incubated in the absence or presence of protein p4 (830 nM) with either nonreconstituted RNAP (indicated as WT-NR), with an RNAP reconstituted either with wild-type $\alpha$ subunit (indicated as $\alpha$WT), or with the $\alpha$ mutant derivative lacking the last 15 residues (indicated as $\alpha$ $\Delta$15). Equivalent amounts of RNAP were added in all cases in terms of specific activity. The complexes formed were resolved through a band-shift gel. FD, free DNA; p4, protein p4–DNA complex; and p4+RNAP, protein p4-RNAP–DNA complex.

footprint generated by protein p4, suggesting that the $\alpha$ subunit binds to a site that partially overlaps protein p4 binding site (Fig. 2). Protein p4 generates a DNase I footprint at the promoter from positions −58 to −104 relative to the transcription start site (5). In the presence of the $\alpha$ subunit, additional protections were observed at positions −58 and −65. These protections were not seen when protein p4 was absent or when it was substituted by mutant protein R120Q, which confirms that the $\alpha$ subunit by itself cannot bind to the promoter, and that protein p4 residue Arg-120 is essential for the p4-mediated binding of the $\alpha$ subunit to the promoter. It should be noted that the footprinting assay does not distinguish whether the $\alpha$ subunit protections are due to a direct interaction with DNA or they appear because, even if there is not an $\alpha$ subunit–DNA interaction, the $\alpha$ subunit hinders the access of DNase I to DNA. Alternatively, the $\alpha$ subunit may be inducing a conformational change in protein p4 that is reflected in a modification of the footprint.

**Deletion of the Last 15 residues of the $\alpha$ Subunit Abolishes Its Ability to Bind to the Promoter in the Presence of Protein p4.** The carboxyl-end of RNAP $\alpha$ subunit has two functions in transcription initiation: (*i*) it acts as the receiver domain for interactions with certain transcription activators (16), and (*ii*) it exhibits sequence-specific DNA binding activity (18, 23–25). Specific binding is observed only at those promoters containing an UP element, an A+T-rich sequence located at the −40 to −70 region that considerably enhances promoter efficiency (23–25). Deletion of the last 73 amino acids of the $\alpha$ subunit of *E. coli* RNAP eliminates both its ability to interact with transcription factors (16) and to bind to UP elements (23). Because $\alpha$ subunit deletion mutants have been described only for the *E. coli* protein, and protein p4 does not interact with *E. coli* RNAP (6), we performed a series of nested deletions at the carboxyl-end of the *B. subtilis* RNAP $\alpha$ subunit to test its role in protein p4-mediated transcription activation.

Mutant proteins lacking the last 15, 37, and 59 residues of the $\alpha$ subunit carboxyl-end were obtained by introducing a stop codon at the corresponding positions of the *B. subtilis rpo*A gene (see Fig. 3). Because *B. subtilis* $\alpha$ subunit is 11 residues shorter than the *E. coli* $\alpha$ subunit, and taking into account that both proteins share an homology at the carboxyl-end high enough to allow a confident alignment of their amino acid sequences (36% identity/65% similarity in the region shown in Fig. 3), the *B. subtilis* mutant protein lacking the last 59 residues is similar to the *E. coli* $\alpha$ subunit derivative lacking the

last 73 amino acids, the shortest deletion described for the *E. coli* α subunit (Fig. 3). The three mutant proteins were purified, and their ability to bind to the A3 promoter in the presence of protein p4 was assayed by gel retardation. The results indicate that the last 15 residues of *B. subtilis* α subunit are needed for its p4-mediated binding to the promoter (Fig. 4). It has been described that a reconstituted *E. coli* RNAP lacking the last 73 residues from the α subunit carboxyl-end is able to transcribe from constitutive promoters, but can no more respond either to UP elements or to activators interacting with α (16, 23, 32). To test if this was also the case with the *B. subtilis* α subunit mutant derivatives, we reconstituted the RNAP holoenzyme with the mutant α subunits and analyzed the interaction of the reconstituted RNAPs with protein p4.

**Behavior of Reconstituted RNAP with Deletions at the α Subunit Carboxyl-End.** RNAP holoenzymes with mutant α subunits were obtained as indicated. Reconstituted RNAPs lacking the last 15, 37, or 59 residues from the α subunit carboxyl-end could recognize phage φ29 promoters not activated by protein p4 (data not shown). Nevertheless, protein p4 could not stimulate transcription from the A3 promoter with these mutant reconstituted RNAPs, while stimulation was efficient when wild-type reconstituted RNAP was used (Fig. 5). This indicates that the last 15 residues of the RNAP α subunit are required for protein p4 activation of the A3 promoter. Gel retardation assays showed that protein p4 could stabilize the wild-type reconstituted RNAP at the A3 promoter, but not that containing the α subunit lacking the last 15 residues (Fig. 6). Because protein p4 activates transcription from the A3 promoter by stabilizing the RNAP at the promoter through a direct protein–protein interaction, the results suggest that the interaction between the two proteins is held through the carboxyl-end of the RNAP α subunit. We further analyzed this possibility by performing gel retardation assays with a DNA fragment that contains protein p4 binding site but not that for RNAP at the A3 promoter. This assay has been shown to allow the detection of a complex in which DNA-bound protein p4 is linked to RNAP, probably through protein–protein interaction (6). Fig. 7 shows that wild-type reconstituted RNAP could interact with protein p4 in this assay, but the mutant reconstituted RNAP lacking the last 15 residues from the α subunit carboxyl-end could not. As stated above, the DNase I footprint of the complex formed by the purified α subunit and protein p4 at the A3 promoter suggests that α could be bound at a site that overlaps with protein p4 binding site (see Fig. 2). Therefore, the results of the above band-shift should be interpreted taking into account that the DNA fragment used does contain the sequences that the α subunit protects from DNase I in a p4-dependent way. This would mean that the RNAP in the complex may be attached either to protein p4 or to both protein p4 and DNA. In either case, the assay clearly indicates that the last 15 residues of the α subunit are required for its stable interaction with protein p4.

**Conclusions.** The results described strongly suggest that the interaction between protein p4 and RNAP that stabilizes the RNAP at the late A3 promoter to activate transcription is held between the protein p4 region containing residue Arg-120 and the carboxy-terminal region of the RNAP α subunit. The results also suggest that the activation process could imply a p4-mediated binding of the α subunit carboxy-terminal domain at a DNA region that overlaps or lies just downstream from the activator binding site. Based on the results obtained with *E. coli* RNAP, a general model has been proposed to explain the role of the α subunit in activation (18). Briefly, the α subunit carboxy-terminal domain would make no specific interactions with the DNA at a promoter lacking an UP element but would specifically interact with the UP sequence at those promoters having it, resulting in a higher RNAP–DNA association constant. When the UP element is absent, an interaction between an activator protein and the α subunit carboxy-terminal domain would help to stabilize a nonspecific binding of the α subunit to DNA, thereby increasing the binding constant and/or inducing a change in the RNAP itself. Our results provide a strong evidence in favor of this model and extend its applicability to the distantly related bacteria *B. subtilis*. Indeed, UP elements have been recently found in certain *B. subtilis* promoters (25). Considering the amino acid sequence conservation of α in prokaryotic and chloroplast RNAP (17, 33), our results suggest that the above model may be of general validity in eubacteria.

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