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Novel RNA polymerase σ factor from *Bacillus subtilis*

(promoter recognition/sporulation genes/RNA nucleotidyltransferase)

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ABSTRACT **A modified form of *Bacillus subtilis* RNA polymerase (RNA nucleotidyltransferase) has been isolated that exhibits distinctive transcriptional specificity. This modified enzyme transcribes two cloned genes from the *purA-cysA* region of the *B. subtilis* chromosome whose expression *in vivo* is associated with the process of sporulation. Neither of these genes is transcribed by the usual form of *B. subtilis* RNA polymerase holoenzyme containing a σ factor of 55,000 daltons (σ^{55}). The modified RNA polymerase lacks σ^{55} but contains a newly identified subunit of 37,000 daltons termed σ^{37}. A reconstitution experiment in which σ^{37} was added to core RNA polymerase strongly suggests that σ^{37} is responsible for the transcriptional specificity of the modified RNA polymerase. σ^{37} apparently acts at the level of promoter recognition; this transcriptional determinant enabled core RNA polymerase to form stable binary and ternary ("initiation") complexes with endonuclease restriction fragments containing promoters for the cloned *B. subtilis* genes.**

The first step in transcription of genes in bacteria is the formation of a binary complex of RNA polymerase (RNA nucleotidyltransferase) with promoter sites on DNA (1). The recognition of promoters is catalyzed by a subunit of RNA polymerase known as σ. This polypeptide confers on the core component ($\beta'\beta\alpha_2$) of the bacterial transcriptase the ability to bind at and initiate RNA synthesis from specific sites on the DNA template. The discovery that σ factor governs site selection in bacteria prompted the proposal that different species of σ polypeptide could recognize different classes of promoters (2). Indeed, certain bacteriophage are now known to encode σ-like proteins that direct the transcription of phage genes. In *Bacillus subtilis* phage SP01 (and its close relative SP82), phage-coded σ-like factors replace the host σ factor on the *B. subtilis* transcriptase, thereby converting the bacterial RNA polymerase to a form that recognizes phage "middle" (3–6) and "late" gene promoters (7, 8). The promoters for phage "middle" genes have distinctive nucleotide sequences that differ strikingly from the promoters controlled by the host σ factor (9).

Until now multiple species of σ factor in uninfected bacteria have not been described. Recently, however, we (10) isolated a modified form of *B. subtilis* RNA polymerase that transcribes two cloned *B. subtilis* genes whose transcription *in vivo* is associated with the process of sporulation (11–13). Neither of these genes is transcribed by the usual form of *B. subtilis* RNA polymerase holoenzyme (14, 15) containing a σ factor of 55,000 daltons (σ^{55}). The modified RNA polymerase lacks σ^{55} but contains a newly identified subunit of 37,000 daltons. Here we show by means of a reconstitution experiment that the 37,000-dalton polypeptide (herein termed σ^{37}) is a σ-like factor that confers novel promoter recognition properties on core RNA polymerase. Thus, *B. subtilis* core RNA polymerase interacts with a variety of bacterial and phage-coded σ factors to acquire multiple transcriptional specificities.

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MATERIALS AND METHODS

^{32}P-Labeled RNA was synthesized in 50-μl reaction mixtures containing 40 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.25 mg of bovine serum albumin per ml, and 10% (vol/vol) glycerol (10). In the reconstitution experiments, core RNA polymerase and σ^{55} and σ^{37} were incubated for 5 min at 0°C prior to addition of template. After the addition of plasmid DNA (2.5 μg), the reaction mixtures were incubated for 10 min at 37°C. RNA synthesis was then initiated by the addition of 0.15 mM CTP, ATP, and GTP and 0.4 μM (5 μCi; 1 Ci = 3.7×10^{10} becquerels) [α-^{32}P]UTP. After incubation for 4 min at 37°C, unlabeled UTP (0.15 mM) was added and the reaction was continued for an additional 2 min. ^{3}H-Labeled RNA was synthesized as described above except that 50 μM (3 μCi) [^{3}H]UTP was substituted for the [^{32}P]UTP and the reaction mixture was incubated for 10 min at 37°C.

RESULTS

Physical Map of Cloned DNA. We used cloned DNA from the *purA-cysA* region of the *B. subtilis* chromosome as a template for *in vitro* RNA synthesis. This cloned chromosomal segment contains two vegetative genes (the *Veg* gene and the *Tms* gene) and two genes [the 0.4-kb (kilobase) gene and the *Ctc* gene] whose transcription appears to be under sporulation control (refs. 11–13 and unpublished data). The *Veg* gene and the *Tms* gene are transcribed *in vitro* by RNA polymerase containing σ^{55}. The 0.4-kb gene and the *Ctc* gene, on the other hand, are uniquely transcribed by RNA polymerase containing σ^{37}.

The location and direction of transcription of these four genes are shown in the endonuclease restriction map of Fig. 1. The cloned chromosomal segment is carried on two hybrid plasmids of the *Escherichia coli* vector pMB9 that contain overlapping *B. subtilis* DNA inserts. p213 contains the *Veg* gene and most of the 0.4-kb gene, whereas p63 contains the 0.4-kb gene, the *Tms* gene, and the *Ctc* gene. Together the DNA inserts in p213 and p63 span an 8-kb segment of the *B. subtilis* chromosome.

Purification of σ^{37}. To test the idea that σ^{37} was responsible for the novel transcriptional specificity of modified RNA polymerase, we attempted to isolate σ^{37} free of core RNA polymerase and then reconstitute the σ^{37}–enzyme complex. σ^{37}-Containing RNA polymerase is displayed in the Na-DodSO$_4$/polyacrylamide slab gel of Fig. 2*A Left*; the purified enzyme contained β′, β, α, and σ^{37} in an approximate stoichiometry of $\beta'\beta\alpha_2\sigma^{37}$ as well as trace amounts of several other polypeptides. As a demonstration of specific transcription by this modified polymerase, RNA was transcribed *in vitro* from p63 and p213 DNAs and then hybridized to electrophoretically separated endonuclease restriction fragments of the corre-

Abbreviation: kb, kilobase.

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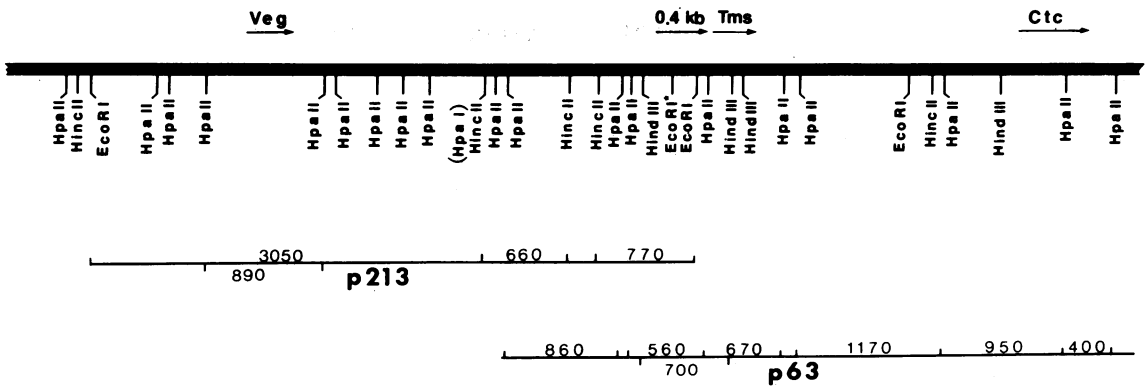

FIG. 1. Endonuclease restriction map of cloned *B. subtilis* DNA. The thick line is a cleavage map for the indicated endonuclease restriction enzymes. The positions on the physical map of the *B. subtilis* DNA inserts in plasmids p213 and p63 are shown at the bottom of the figure. The sizes of certain *Eco*RI/*Hin*cII and *Hpa* II fragments are indicated in base pairs, respectively, above and below the p213 insert line. Similarly, certain *Hpa* II and *Hin*dIII fragments are identified, respectively, above and below the p63 insert line. The location and orientation of the *Veg*, 0.4-kb, *Tms*, and *Ctc* genes are indicated above the physical map. The right-hand end points of the *Tms* and *Ctc* genes are uncertain. The information in this figure is from refs. 10 and 12 and unpublished data.

sponding plasmid DNA. These Southern hybridizations showed that σ^{37}-containing RNA polymerase selectively transcribed RNA from a DNA segment (*Hpa* II-950) of p63 that contains the *Ctc* gene (Fig. 2*C Left*) and a p213 DNA segment (*Eco*RI/*Hin*cII-770) that contains the 0.4-kb gene (Fig. 2*D Left*).

To purify σ^{37}, we denatured the modified RNA polymerase in 6 M urea and then applied the denatured enzyme to a column of phosphocellulose. Elution with a linear gradient of KCl completely separated σ^{37} from α polypeptide in the flow-through volume) and β' (which remained bound to the column) and partially separated σ^{37} (eluting at 0.16 M KCl) from β (eluting at 0.09 M KCl) and several proteolytic fragments of β that were generated during the denaturation step (Fig. 2*A*).

After removal of the urea by dialysis, samples from the gradient elution were added to native core RNA polymerase (15, 17) and tested for their ability to stimulate RNA synthesis with p63 DNA as template or to direct selective transcription of the *Ctc* and 0.4-kb genes. (In the absence of core RNA polymerase these samples exhibited no detectable RNA polymerizing activity.) As shown in Fig. 2*B*, fractions containing σ^{37} polypeptide strongly stimulated transcription of p63 DNA. Likewise, as judged by Southern hybridizations, stimulation of *Ctc* gene (Fig. 2*C*) and 0.4-kb gene (Fig. 2*D*) transcription was coincident with the peak fraction (number 10) for σ^{37} content. This pattern of stimulation was observed with core RNA polymerase from either vegetative or sporulating cells. Thus, selective RNA synthesis from p63 DNA was strongly correlated with the σ^{37} polypeptide.

Specificity of σ^{37}-Directed Transcription. The isolation of the 37,000-dalton transcriptional determinant made it possible to compare directly the effects of σ^{55} and σ^{37} on site selection by core RNA polymerase. Fig. 3 shows that both proteins strongly stimulated transcription of p63 DNA by core RNA polymerase although neither transcriptional determinant markedly enhanced RNA synthesis from the plasmid vector DNA pMB9. Hybridization of RNA synthesized *in vitro* to separated endonuclease restriction fragments revealed, however, that σ^{55} had stimulated transcription from a DNA segment (*Hpa* II-670) that contains the *Tms* gene (Fig.. 3*B Inset*) whereas σ^{37} had caused transcription from a segment (*Hpa* II-950) that contains the *Ctc* gene (Fig. 3*A Inset*). Thus, each factor had directed specific transcription from distinct regions of the hybrid plasmid DNA.

[Notice, however, that even though p63 contains the 0.4-kb gene (see Fig. 1), the inset in Fig. 3*A* does not reveal hybridization to the *Hpa* II 560-base-pair fragment that contains the 0.4-kb gene. In other work we have shown that the *Ctc* gene or DNA associated with it strongly inhibits transcription of the 0.4-kb genes, although at high polymerase concentrations transcription of both genes can be detected. The *Ctc* promoter is apparently a much stronger initiation signal for modified polymerase than is the 0.4-kb gene promoter.]

Do σ^{55} and σ^{37} promote specific RNA synthesis from unique sites on p63 DNA? To answer this question we used as a template p63 DNA that had been cut with the restriction endonucleases *Hin*dIII and *Hpa* II. σ^{55} directed the synthesis of an 85-base RNA from the truncated DNA template whereas σ^{37} caused polymerase to synthesize a 365-base RNA (not shown). The 85-base RNA was generated by "run-off" transcription from a *Hin*dIII site within the *Tms* gene whereas the 365-base RNA was generated by "run-off" RNA synthesis from a *Hpa* II site within the *Ctc* gene (see Fig. 1). Neither "run-off" transcript was synthesized by core RNA polymerase in the absence of added factor. Combined with the experiment of Fig. 3, these results show that σ^{55} and σ^{37} cause RNA polymerase to initiate at unique and discrete sites on p63 DNA.

RNA Polymerase–DNA Fragment Binding Complexes. RNA polymerase σ factors enable core enzyme to recognize and bind to promoter sites on DNA (1, 5). Does σ^{37} work at the level of promoter recognition by causing RNA polymerase to bind to the transcription initiation sites for the *Ctc* and 0.4-kb genes? To investigate this question, we examined the ability of σ^{37}-containing RNA polymerase to form stable binary complexes with *Hpa* II fragments of p63 DNA. Fragments of DNA that can form such stable binding complexes with RNA polymerase can be isolated by their ability to be retained on nitrocellulose filters (18, 19). Therefore, if σ^{37} acts at the level of promoter recognition, then in the absence of nucleoside triphosphates σ^{37}-containing RNA polymerase ought to bind tightly to, and hence retain on nitrocellulose, the *Hpa* II-950 fragment that contains the *Ctc* gene promoter.

Radioactively labeled fragments of *Hpa* II-cut p63 DNA were incubated under binding conditions with core RNA polymerase, σ^{37}, or a mixture of core polymerase and σ^{37}. After filtration of the reaction mixtures, DNA fragments that were retained on the nitrocellulose were eluted with $NaDodSO_4$ and displayed by electrophoresis through agarose. The principal *Hpa* II fragments of p63 are displayed in track A of Fig. 4. In the presence of core polymerase alone (track B) or σ^{37} alone

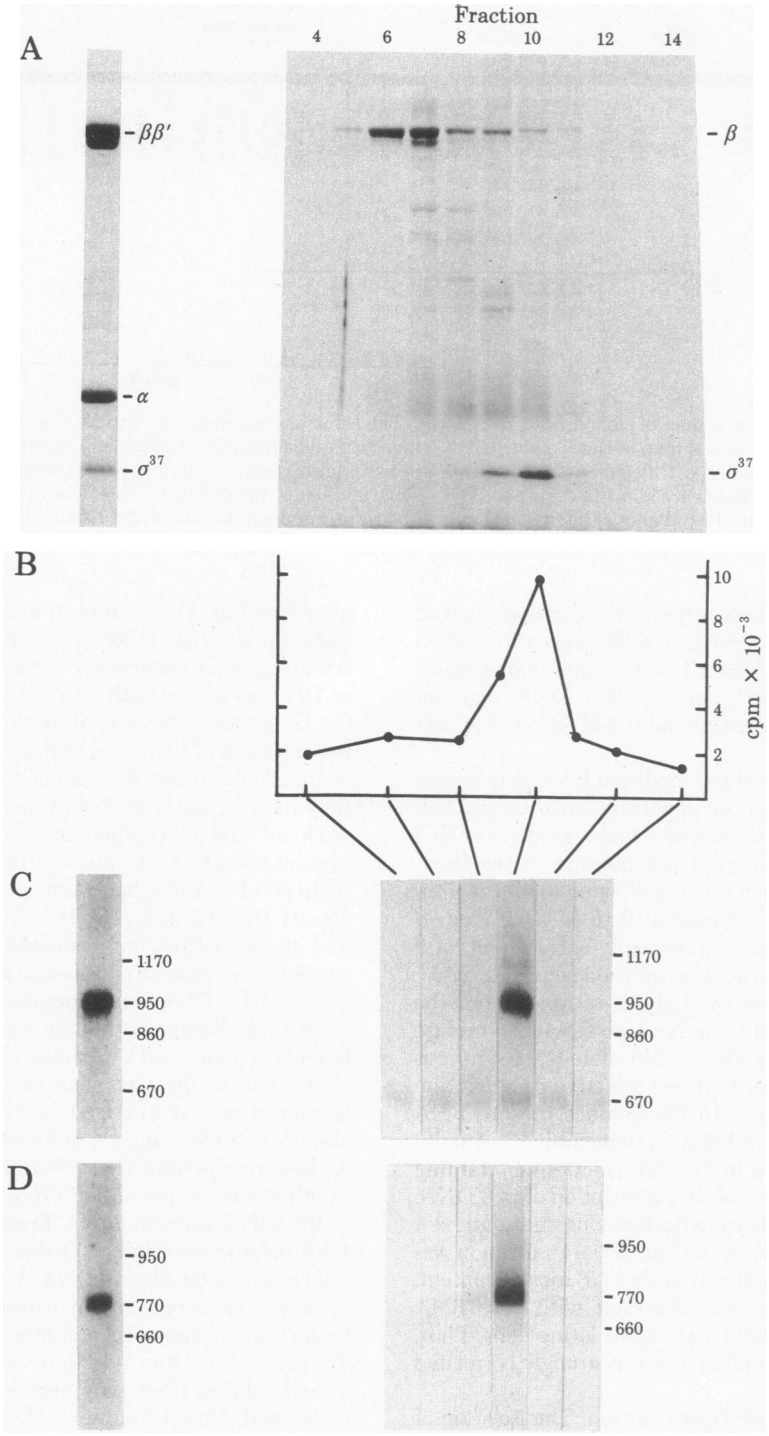

FIG. 2. Separation of σ^{37} from core RNA polymerase by phosphocellulose chromatography. A 0.5-mg sample of σ^{37}-containing RNA polymerase (10) was denatured in buffer D (50 mM Tris·HCl, pH 8.0/0.1 mM EDTA/0.4 mM dithiothreitol) containing 6 M urea, 50 mM KCl, and 10 mg of hemoglobin per ml, applied to a 2-ml phosphocellulose column that had been equilibrated with buffer D containing 6 M urea and 50 mM KCl, washed with this buffer until hemoglobin was no longer detectable in the flow-through volume, and then eluted with a 75-ml linear gradient of KCl (0.05–0.5 M) in buffer D containing 6 M urea. Fractions of 2 ml were collected and dialyzed individually against renaturation buffer [50 mM Tris·HCl, pH 8.0/0.1 mM EDTA/8 mM dithiothreitol/0.3 M KCl/50% (vol/vol) glycerol]. (*A*) $NaDodSO_4$ slab-gel electrophoresis. (*Left*) σ^{37}-containing RNA polymerase; (*Right*) 200-μl samples of dialyzed fractions from the gradient elution of urea-denatured enzyme from phosphocellulose columns were subjected to electrophoresis through a slab gel containing $NaDodSO_4$ in a Tris/glycine buffer and a 10–20% gradient of polyacrylamide. The gel was stained with Coomassie brilliant blue. (*B*) Stimulation of transcription. ^{32}P-Labeled RNA was transcribed *in vitro* from p63 DNA by 0.01 unit of core RNA polymerase [1 unit of RNA polymerase incorporates 1 nmol of UMP in 10 min at 37°C with poly(dA-dT) as template] to which samples (0.5 μl) of dialyzed fractions from the gradient elution had been added. RNA synthesis was measured as trichloroacetic acid-insoluble radioactivity. (*C* and *D*) Southern hybridization. ^{32}P-Labeled RNA was transcribed *in vitro* from p63 DNA (*C*) or from p213 DNA (*D*) by either σ^{37}-containing RNA polymerase (0.3 μg; *Left*) or by core RNA polymerase (0.01 unit) to which dialyzed samples (0.5 μl) had been added from alternate fractions of the phosphocellulose column. The RNAs synthesized *in vitro* were then incubated under hybridization conditions (10) with nitrocellulose strips (16) containing electrophoretically separated *Hpa* II fragments of p63 (*C Right*) or *Eco*RI/*Hin*cII fragments of p213 DNA (*D Right*). Only fragments in the vicinity of the *Hpa* II-950 fragment of p63 or the *Eco*RI/*Hin*cII-770 fragment of p213 are shown.

(track C), little retention of any of the fragments on nitrocellulose could be detected. However, a mixture of both core polymerase and σ^{37} caused preferential retention of the *Hpa* II-950 fragment. In an analogous experiment with a subclone of p63 that contains the 0.4-kb gene, σ^{37} also directed the formation of a stable binary complex with a restriction fragment containing the 0.4-kb gene promoter.

Is the binding site on the *Hpa* II 950-base-pair fragment a promoter from which polymerase can initiate transcription? To answer this question we investigated whether brief exposure to ribonucleoside triphosphates would convert the binary complexes to "initiation" complexes of enzyme, DNA fragment, and nascent RNA chains (20). Such ternary complexes are extremely stable and can be detected by filtration through nitrocellulose membranes after incubation at low temperature (0°C) and high ionic strength (0.25 M KCl), conditions that dissociate binary complexes of RNA polymerase and DNA promoters. Track E of Fig. 4 shows that the binary complex formed in the absence of ribonucleoside triphosphates was unable to retain *Hpa* II-950 DNA on nitrocellulose after incubation at low temperature and high ionic strength. However, in the presence of ATP, GTP, UTP, and CTP, σ^{37}-containing RNA polymerase formed a complex with *Hpa* II-950 DNA that was stable under the conditions that dissociated the binary complex (Fig. 4, track F). We conclude, therefore, that σ^{37} directs RNA polymerase to bind to a site on *Hpa* II-950 DNA from which RNA synthesis can be initiated. This site is presumably the promoter for the *Ctc* gene.

DISCUSSION

We have distinguished two forms of *B. subtilis* RNA polymerase that exhibit distinct transcriptional specificities. The usual form of RNA polymerase holoenzyme containing σ^{55} actively transcribed RNA from the cloned DNA of the *Veg* gene (10) and the *Tms* gene but failed to transcribe measurably either the 0.4-kb gene or the *Ctc* gene. In contrast, modified RNA polymerase containing σ^{37} selectively transcribed the 0.4-kb and *Ctc* genes. We believe that σ^{37} is itself responsible for this transcriptional specificity; the novel transcribing activity of modified polymerase copurified with enzyme-bound, 37,000-dalton polypeptide during phosphocellulose and DNA cellulose chromatography of the native enzyme (10) and, in a reconsitution experiment, with free 37,000-dalton polypeptide purified by phosphocellulose chromatography of urea-denatured enzyme (Fig. 2).

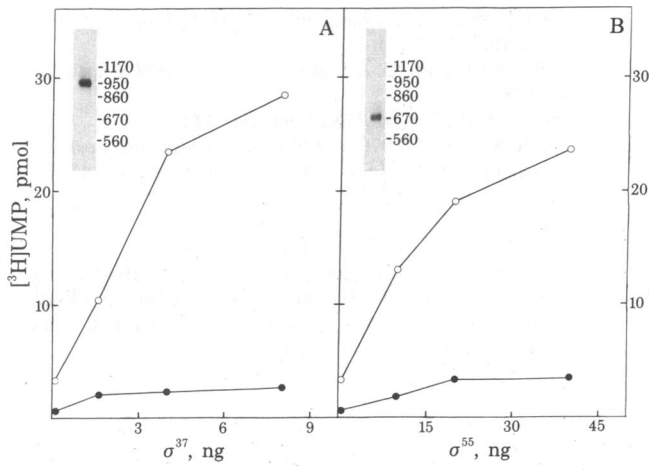

FIG. 3. Stimulation of specific transcription by purified σ factors. ^{3}H-Labeled RNA was transcribed *in vitro* from p63 DNA (○) or pMB9 DNA (●) by core RNA polymerase (0.01 unit) to which the indicated amounts of σ^{37} (*A*) or σ^{55} (*B*) had been added. RNA synthesis was measured as trichloroacetic acid-insoluble radioactivity. (*Insets*) Southern hybridizations of ^{32}P-labeled RNA transcribed *in vitro* to nitrocellulose strips containing *Hpa* II fragments of p63 DNA. The ^{32}P-labeled RNA was synthesized by core RNA polymerase (0.01 unit) and either 4 ng of σ^{37} (*Inset A*) or 20 ng of σ^{55} (*Inset B*).

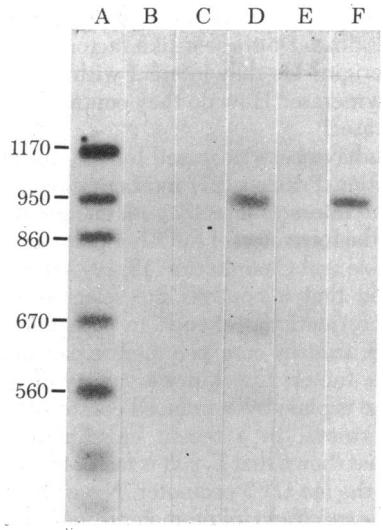

FIG. 4. Binding of RNA polymerase to DNA restriction fragments. ^{32}P-End-labeled fragments of Hpa II-cut p63 DNA were incubated in a 25-μl binding reaction mixture (8, 9) with core RNA polymerase (0.02 unit/pmol of DNA) alone (track B), σ^{37} (12 ng) alone (track C), or a mixture of core RNA polymerase and σ^{37} (track D) at 37°C for 10 min. The reaction was terminated by addition of 5 μg of calf thymus DNA, and the mixture was incubated for an additional 10 min at 37°C. The binding reaction mixtures were then diluted to 500 μl with binding buffer (8) that had been warmed to 37°C. Stable binary complexes were isolated by filtration through nitrocellulose. DNA fragments were then eluted from the filters with $NaDodSO_4$ and subjected to electrophoresis through agarose. (Track E) ^{32}P-Labeled fragments were treated with core RNA polymerase and σ^{37} as for track D, but the binary complexes were then incubated at 0°C and high ionic strength (0.25 M KCl) for 30 min prior to filtration through nitrocellulose. (Track F) Binary complexes were formed by reaction of ^{32}P-labeled fragments with core RNA polymerase and σ^{37} as for track D, but GTP, ATP, CTP, and UTP (0.15 mM) were added along with calf thymus DNA. Initiation complexes were formed by incubating the reaction mixture for 1 min at 37°C and were then selected by incubation at 0°C with 0.25 M KCl for 30 min prior to filtration through nitrocellulose (8). (Track A) ^{32}P-Labeled *Hpa* II fragments are shown. The radioactive fragments were visualized by autoradiography.

Like σ^{55}, σ^{37} appears to act at the level of promoter recognition; it conferred on core RNA polymerase the ability to recognize and bind to sites at or near the promoters for the *Ctc* and 0.4-kb genes. Thus, σ^{37} should itself be considered an RNA polymerase σ factor of novel promoter specificity. In contrast to numerous reports of modified forms of bacterial RNA polymerase (17, 21–25), in this case distinctive transcriptional specificities can be attributed to separate σ factors within a single bacterium. It is likely that σ^{37} and σ^{55} are the products of separate genes because we (unpublished results) have found that σ^{37} is resistant to antibodies against σ^{55} (26). However, a definitive investigation of this will require endopeptidase analysis or the identification of the structural gene(s) for these two polypeptides.

The discovery of σ^{37} extends to four the list of σ-like factors with which *B. subtilis* core RNA polymerase can interact. In addition to the bacterial σ factors σ^{55} and σ^{37}, phage SP01 (and its close relative SP82) encode two σ-like factors that control phage "middle" and "late" gene transcription: gp28, the product of regulatory gene 28 (3–5), and the synergistic pair gp33 and gp34, the products of regulatory genes 33 and 34 (7,

8). This versatility of *B. subtilis* core polymerase poses several intriguing questions. Do these σ-like factors have a common evolutionary origin? Do they interact with a common site on core RNA polymerase? How do they control site selection by RNA polymerase?

Two models have been proposed for the role of σ factor in DNA site selection. Pribnow (27) and Losick and Pero (28) have proposed that σ interacts directly with nucleotides in the promoter during the formation of the RNA polymerase–promoter complex. Hinkle and Chamberlin (18, 19), on the other hand, have suggested that σ controls site selection indirectly by causing a conformational change in core polymerase. The finding that *B. subtilis* core polymerase can interact with a series of σ-like factors to acquire a variety of specificities is cumbersome to explain by σ-induced conformational changes alone. Furthermore, in a recent crosslinking experiment, Simpson (29) has shown that *E. coli* σ factor touches at least one nucleotide in the *lac* UV5 promoter. Nevertheless, a demonstration that the *specificity* of promoter recognition is controlled by direct interaction of σ with promoter sequences is still lacking. It is, of course, not excluded that site selection could be controlled by a combination of direct and indirect effects of σ on promoter recognition.

What is the physiological function of σ^{37}? The expression of the 0.4-kb gene and the *Ctc* gene is associated with the process of spore formation in *B. subtilis*. Both genes are transcribed at only a low level in vegetative cells but are actively transcribed in sporulating bacteria. Moreover, a mutation (*spoVC285*; ref. 30) that maps within or very near the *Ctc* sequence (ref. 13 and C. P. Moran, personal communication) blocks the sporulation process. It might thus be expected that σ^{37} is a regulatory factor controlling sporulation-specific transcription. Several observations suggest, however, that σ^{37} may be only one element in a complex regulatory pathway that controls 0.4-kb and *Ctc* RNA synthesis. First, under certain growth conditions σ^{37} is detected in vegetative cells (31) even though both genes are transcribed at a low rate during growth. Second, transcription *in vivo* of the 0.4-kb gene but not the *Ctc* gene is dependent upon the products of five *B. subtilis spo0* genes, regulatory cistrons that are involved in the initiation of sporulation, even though σ^{37} directs the transcription of both genes *in vitro* (unpublished observations). Third, σ^{37} is not the only protein that directs *Ctc* transcription; a form of *B. subtilis* polymerase containing a sporulation-induced subunit of 29,000 daltons (23, 24) is also capable of transcribing the *Ctc* sequence (unpublished data). The role of σ^{37} in regulating gene expression in *B. subtilis* is thus unclear. Determination of the function of this protein must await the isolation of mutations in the σ^{37} structural gene and an analysis of their resulting phenotypes.

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