Changes in the Association between *Bacillus subtilis* RNA Polymerase Core and Two Specificity-Determining Subunits during Transcription†

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ABSTRACT: The *Bacillus subtilis* RNA polymerase σ subunit and the phage SPO1-coded gene 28 protein are responsible for selective binding of RNA polymerase to early and middle SPO1 promoters, respectively. The association of the RNA polymerase core with each of these subunits weakens during the elongation of RNA chains. Similar changes are known to be an essential part of the *Escherichia coli* RNA polymerase σ cycle.

Several aspects of the association of *Escherichia coli* RNA polymerase with DNA change in characteristic ways during transcription [for reviews, see Siebenlist et al. (1980) and Chamberlin (1976)]. In particular, the affinity of the σ subunit for the RNA polymerase core changes at an early stage of RNA chain elongation (Hansen & McClure, 1980). During the development of the *Bacillus subtilis* bacteriophage SPO1, the regulation of gene expression is under the positive control of the products of three viral genes which code for RNA polymerase binding proteins. *B. subtilis* RNA polymerase containing these subunits in place for σ selectively binds to specific sites on SPO1 DNA which most probably are promoters for so-called middle and late genes [for a recent review, see Geiduschek & Ito (1981)]. As a consequence of studies on the SPO1 gene 28 protein containing RNA polymerase, which selectively transcribes viral middle genes (Duffy & Geiduschek, 1973, 1977), we have become interested in the fate of gp28 during transcription. In addition, the fate of *B. subtilis* σ during transcription has been the subject of conflicting reports (Williamson & Doi, 1979; Spiegelman & Whiteley, 1979). In the work that is reported here, we have analyzed the protein contents of transcription complexes which have been separated from unbound components by centrifugation. The composition of the protein in these complexes has been analyzed by gel electrophoresis, taking advantage of recent improvements in the sensitivity of detecting protein by staining with silver (Switzer et al., 1979).

The experiments demonstrate that the affinity of gp28 subunit for the *B. subtilis* RNA polymerase core is decreased during transcription. Furthermore, our results indicate that the *B. subtilis* σ is released during transcription, as Spiegelman & Whiteley (1979) contended.

Materials and Methods

Materials. *B. subtilis* RNA polymerase holoenzyme (E.σ) and RNA polymerase from phage SPO1 *sus* F4-F14-infected *B. subtilis* 168M (su^-), collected 12 min after infection at 37 °C, were prepared as outlined and referenced previously (Chelm et al., 1981). The latter is the so-called middle enzyme (E.gp28) which has a phage-coded 28 000-dalton subunit (gp28; Duffy & Geiduschek, 1975; Fox et al., 1976) bound to *B. subtilis* RNA polymerase core. E.gp28 selectively transcribes phage SPO1 middle genes (Duffy & Geiduschek, 1973, 1975; Pero et al., 1975). Two preparations of *E. coli* RNA polymerase were used. One sample, prepared by a combination of published methods (Zillig et al., 1970; Burgess & Jendrisak, 1975), was the generous gift of D. P. Rabussay. A second preparation (Gonzalez et al., 1977) was the generous gift of G. Kassavetis. Concentrations of RNA polymerase were measured according to Lowry et al. (1951) by using bovine serum albumin as standard. Gp28 was prepared from purified E.gp28 by slight modifications of a previously published method (Duffy & Geiduschek, 1977), which will be described elsewhere. The particular preparation used here also contained some contaminating RNA polymerase α subunit. *B. subtilis* σ subunit was prepared from E.σ by a previously published method (Duffy & Geiduschek, 1977). T7 and SPO1 DNAs were prepared from purified phage by phenol extraction (Mandell & Hershey, 1970). A sample of SPO1 DNA with long single-stranded ends was prepared as follows. Sixty-five grams of SPO1 DNA was digested for 90 min with *E. coli* exonuclease III (New England Biolabs, Beverly, MA; 1 unit generates 1 nmol of nucleotide from 3′ termini of DNA in 30 min under the conditions specified by the supplier) in 25 mM Tris-HCl,[1] pH 8.0, 0.25 mM $MgCl_2$, and 5 mM β-mercaptoethanol at 37 °C. The exonuclease was added in three successive 100-unit aliquots at 30-min intervals. SPO1 DNA 3′ ends are removed at approximately 160 nucleotides/min (J. Romeo, personal communication) under the above conditions.

Formation, Separation, and Analysis of RNA Polymerase–DNA Complexes. DNA (1–6 μg) and RNA polymerase (0.4–2.4 μg) were incubated at 30 °C for 10 min in 30 μL of a reaction buffer containing 100 mM Tris-H Cl, pH 8.0, 10 mM $MgCl_2$, 50 μg/mL bovine serum albumin [Pentex, Kankakee, IL; dissolved in water at 10 mg/mL and centrifuged in the Airfuge (see below) at 29 psi of air pressure for 30 min before use], 0.1 mM dithiothreitol, and 0.1 mM EDTA in order to form binary complexes. For ternary complex formation, 10 μL of ribonucleoside triphosphates in reaction buffer was added to a final concentration of 500 μM each of ATP and GTP and 5 μM each of CTP and UTP, and the reaction mixture was further incubated at 30 °C for 2 or 4 min. The reaction was stopped by adding 10 μL of 100 mM NaEDTA, pH 8, also containing 0, 250, or 500 μM aurintricarboxylic acid (ATA). The mixture was chilled on ice for 4 min and then layered on a cushion consisting of 100 μL of

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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25% glycerol in reaction buffer lacking bovine serum albumin and containing the appropriate "stop" (i.e., 20 mM EDTA or 20 mM EDTA with 100 μM ATA) in a microcentrifuge tube (cellulose propionate, 5 × 20 mm, 175 μL total volume; Beckman, Fullerton, CA). Centrifugation was carried out in a Beckman Airfuge by using an A-100 fixed angle rotor at 29 psi of air pressure [95 000 rpm; $(1.5 \times 10^5)g_{max}$] for 30 min at room temperature. The time of centrifugation was chosen on the basis of the time required to sediment 5 μg of SPO1 DNA from the 50-μL sample through the cushion to the bottom of the centrifuge tube. After centrifugation, the top two-thirds of the tube was cut off, and the remaining supernatant was removed with a pipet. The pelleted DNA and protein were resuspended in 25 μL of 10 mM Tris-HCl, pH 6.8, 1–5 mM dithiothreitol, 1% sodium dodecyl sulfate ($NaDodSO_4$), 0.1% bromophenol blue, and 10% (v/v) glycerol and heated at 100 °C for 1 min before being loaded on a 15% acrylamide low cross-linking (acrylamide:bisacrylamide 150:1) $NaDodSO_4$ gel for discontinuous electrophoresis according to Laemmli (1970) (cutting off the top of the centrifuge tube eliminated contamination with protein from the supernatant phase, a certain amount of which sticks to the side of the centrifuge tube, and is then eluted with the sodium dodecyl sulfate when the pelleted polymerase–DNA complex is resuspended for gel electrophoresis).

Gels were silver stained, using the original method of Switzer et al. (1979). Stained gels were scanned on a Joyce Loebl densitometer, with each lane of the gel being measured several times with successive lateral displacements and averaged. The stain density associated with bands of RNA polymerase subunits was quantitated from the plotted density profiles by measuring areas under the appropriate peaks with a Hewlett-Packard digitizer interfaced with a Hewlett-Packard 9821A desk top calculator, using a program kindly prepared by M. M. Miller.

With each gel, containing several appropriate experimental and control samples, we also included four different aliquots of the apropriate RNA polymerase, which served as internal standards for the subsequent staining and quantitation. Stain density associated with the α, σ, and gp28 subunits was primarily used in subsequent calculations. (One avoids any possible complications of differential staining of these three proteins by including the RNA polymerase standards.) We did not quantitate β and β′ subunits because these bands lie in an area of the gel in which we frequently found substantial background density and because these very sharp bands, which contain relatively large amounts of protein, were saturated with stain under our conditions. Nor have we quantitated the subunits which have usually been electrophoresed off the gel.

In one series of centrifugation experiments, DNA was quantitated by the DAPI (4′,6-diamido-2-phenylindole hydrochloride) fluorescence method of Kapuscinsky & Skoczylas (1977). DNA remaining in the pellet after centrifugation was redissolved in 0.5 mL of 10 mM Tris-HCl, pH 8.0, by shaking overnight at 4 °C. Three successive 15-μL aliquots of each redissolved DNA sample were added to 2 mL of 30 ng/mL DAPI in 10 mM Tris-HCl, pH 8, and 10 mM NaCl in a cuvette placed in a spectrofluorometer. The sample was stirred after each DNA addition and illuminated at 342 nm, and the fluorescence intensity was measured at 454 nm. A standard curve was constructed in the same way with SPO1 DNA.

Results

Transcription Complexes in the Presence of Aurintricarboxylic Acid. Aurintricarboxylic acid (ATA) inhibits RNA polymerase. When added before initiation of RNA synthesis, 1.5–4 μM ATA inhibits *E. coli* and *B. subtilis* enzymes 50% (Table I). Once RNA synthesis has started, the sensitivity of inhibition decreases drastically. Samples of 50 and 100 μM ATA barely change the RNA chain elongation rate with *E. coli* E.σ (data not shown). *B. subtilis* E.σ is somewhat more sensitive to inhibition of RNA chain elongation by ATA, but the inhibition is reversible, as the following experiment (Figure 1) shows. SPO1 RNA synthesis was allowed to start; 2 min later, further initiation was blocked with 25 μg/mL rifampicin. ATA was added at the same time: to 12.5 μM in sample 2 and to 50 μM in sample 1. After another 8 min, sample 1 was further split, with one aliquot being diluted 4-fold into 25 μg/mL rifampicin so that the final concentration of ATA was 12.5 μM (sample 3), while another aliquot was diluted 4-fold into 25 μg/mL rifampicin and, 50 μM ATA (sample 4). A 50 μM sample of ATA lowers the rate of UTP incorporation by about 55% (relative to 12.5 μM), but the incorporation rate recovers when ATA is diluted out. Similar results have been obtained with E.gp28, the SPO1-modified middle enzyme (data not shown). Since the effect of ATA on RNA synthesis is reversible when reinitiation is blocked by the presence of rifampicin, it does *not* dissociate ternary transcription complexes. That is the property required for the analysis that follows.

Table I: Inhibition of RNA Synthesis Initiation by ATA[a]

ATA (μM)	rel incorporation of UMP by: *E. coli* E.σ-T7 DNA	*B. subtilis* E.σ-SP01 DNA	E.gp28-SP01 DNA
0	(100)	(100)	(100)
0.25	90	94	98
0.5	86	95	89
1	62	95	84
2.5	28	80	49
5	2	36	21
10	<1	1	<1

[a] One microliter of buffer (100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 15% (v/v) glycerol, and 400 μg/mL bovine serum albumin) containing 100 ng of the appropriate RNA polymerase was added on ice to 14 μL of an ATA–DNA mixture to make up 15 μL with the following composition: 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 127 μg/mL bovine serum albumin, 780 ng of SP01 DNA or 224 ng of T7 DNA (for an enzyme, DNA molar ratio of 25:1), and the concentration of ATA specified in the table. After 6 min at 0 °C, RNA synthesis was started by transferring the sample to 30 °C while adding 5 μL of a mixture containing the same concentration of ATA, 2 mM each of ATP, GTP, and CTP, 200 μM [α-^{32}P]UTP, and Tris, $MgCl_2$, EDTA, and dithiothreitol at the above-specified concentrations. RNA synthesis was stopped after 6 min at 30 °C, and incorporation of [α-^{32}P]UTP into RNA was measured by precipitation with Cl_3CCOOH.

ATA dissociates binary complexes of *E. coli* RNA polymerase with T7 DNA under our conditions of preparation and analysis. This is shown by an experiment in which binary complexes were formed at 30 °C in reaction buffer, briefly cooled to 0 °C, and then centrifuged at room temperature, and the protein sedimenting with the T7 DNA was analyzed (Figure 2, lane 1). A separate sample of binary complexes was treated with ATA (final concentration 100 μM). We reasoned that the dissociation of the binary complexes by ATA (Figure 2, lane 2) should be favored by incubation at 0 °C (which converts open to closed promoter complexes). Essentially no RNA polymerase pellets through the glycerol cushion in the absence of DNA (lane 4). On the other hand, ternary complexes are not completely dissociated by ATA (lane 3), but while β, β′, and α are readily detected on the gel, σ is

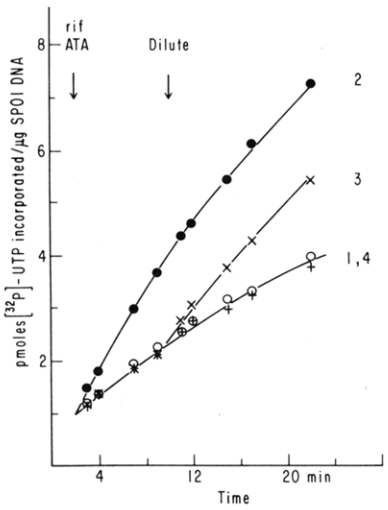

FIGURE 1: Reversible effect of ATA on the rate of RNA chain elongation by *B. subtilis* RNA polymerase. For sample 1 (○), 3.3 μg of SPO1 DNA and 0.5 μg of *B. subtilis* RNA polymerase were incubated at 30 °C in 75 μL of a buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 μg/mL bovine serum albumin, 0.1 mM EDTA, and 0.1 mM dithiothreitol for 10 min. Twenty-five microliters of a mixture containing 50 nmol each of ATP and GTP and 0.5 nmol each of CTP and [α-^{32}P]UTP in the same buffer was then added to start RNA synthesis. Two minutes later, 3.12 μg of rifampicin and 1.56 [sample 2 (●)] or 5 nmol of ATA (sample 1) in 25 μL of the same buffer, also containing all the ribonucleoside triphosphates, were added. After another 8 min, 225 μL of rifampicin–ATA mixture in the same buffer–nucleotide mixture was added to samples 3 (×) and 4 (+). The diluent for sample 3 contained 5.63 μg of rifampicin and no ATA; the diluent for sample 4 contained 5.63 μg of rifampicin and 11.25 nmol of ATA. Aliquots were taken from all four reaction mixtures and assayed for incorporation of [^{32}P]UMP into Cl_3CCOOH-insoluble material.

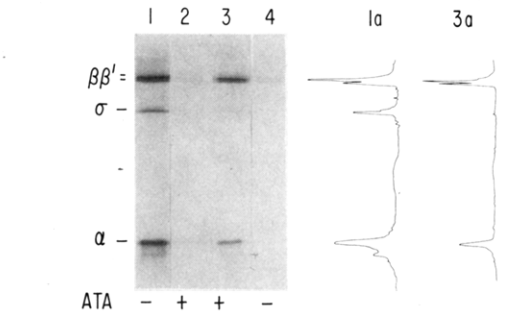

FIGURE 2: Complexes of *E. coli* RNA polymerases with T7 DNA in the presence of ATA. For lanes 1–3, 0.80 μg of *E. coli* RNA polymerase and 2 μg of T7 DNA were incubated as described under Materials and Methods. For lane 4, 0.80 μg of RNA polymerase was mixed with the other components of the standard assay, excepting DNA. For the sample shown in lane 3, ribonucleoside triphosphates were added to the other components, and RNA synthesis was allowed to proceed for 2 min. For lanes 2 and 3, the "stop" mixture contained 500 μM ATA (final concentration 100 μM); for lanes 1 and 4, it did not. All samples were prepared, centrifuged, and electrophoresed as described under Materials and Methods. Densitometric scans of lanes 1 and 3 are shown at the side. These two scans were made with the same density standard (optical wedge), and all other settings of the densitometer were kept constant.

entirely absent. It is well-known that the association of *E. coli* σ with RNA polymerase core changes (Travers & Burgess, 1969) at an early stage of RNA chain elongation. When the nascent RNA chain is less than 10 nucleotides long (Hansen & McClure, 1980), the affinity of σ for the ternary transcription complex decreases sufficiently so that σ can be separated from the other components by various means (Ruet et al., 1970; Wu et al., 1975) and also can be picked up by added RNA polymerase core and thereby recycled for initiating further rounds of transcription. The results shown in Figure 2 are therefore consistent with what is already known about *E. coli* RNA polymerase and serve as orientation and control for the experiments that follow.

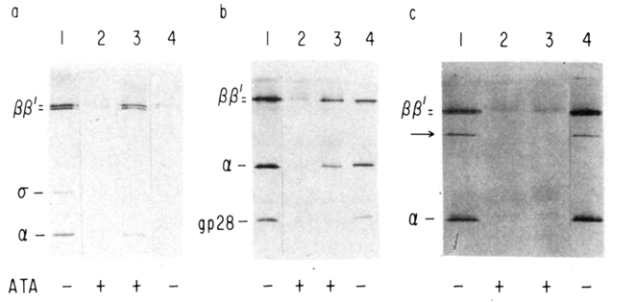

FIGURE 3: Transcription complexes in the presence of ATA. (a) *B. subtilis* RNA Polymerase with SPO1 DNA. For lanes 1–3, 1.06 μg of *B. subtilis* RNA polymerase and 6 μg of SPO1 DNA were incubated as described under Materials and Methods. For lane 4, 1.06 μg of *B. subtilis* RNA polymerase was mixed with the other components of the standard assay, except DNA, on ice. For the sample shown in lane 3, ribonucleoside triphosphates were added to the other components, and RNA synthesis was allowed to proceed for 2 min. For lanes 2 and 3 the "stop" mixture contained 500 μM ATA; for lanes 1 and 4, it did not. (b) Phage SPO1 modified *B. subtilis* middle enzyme (E.gp28) with SPO1 DNA. (Lanes 1–3) A mixture of 2.4 μg of RNA polymerase from SPO1-infected *B. subtilis* (E.gp28) and 5.8 μg SPO1 DNA; (lane 4) 0.4 μg of E.gp28. All other details as in part a. (c) *B. subtilis* RNA polymerase core with SPO1 DNA. A mixture of 1.0 μg of *B. subtilis* core enzyme and 6.0 μg of SPO1 DNA was incubated for the formation of binary complexes (lanes 1 and 2) and ternary complexes (lane 3). ATA (final concentration 100 μM) was present during further incubation of the samples shown in lanes 2 and 3. Preparation and analysis were as described under Materials and Methods. Note that this preparation of core enzyme (lane 4) is contaminated with a 100 000-dalton protein (horizontal arrow) which binds to SPO1 DNA in binary complexes but is removed by ATA; this is consistent with its being either a RNA polymerase-binding protein or an ATA-sensitive DNA-binding protein.

Densitometric scans of Figure 2 (lanes 1 and 3) are also shown. Less DNA-bound RNA polymerase β′, β, and α subunits are present in the ATA-treated ternary complexes than in the corresponding, untreated binary complexes. As discussed further below, this is due to the fact that not all the RNA polymerase molecules which bound to T7 DNA in binary complexes at 30 °C initiated RNA synthesis within the allotted time of 2 min in the presence of 500 μM ATP and GTP and 5 μM CTP and UTP. In experiments with two preparations of *E. coli* RNA polymerase, we noted a qualitative correlation between the fraction of bound RNA polymerase that is retained in the presence of ATA and the fraction of active RNA polymerase molecules, determined according to Chamberlin et al. (1979). Evidently the preparation of RNA polymerase that was used for this experiment contained inactive enzyme molecules that still bind to T7 DNA.

Similar experiments with *B. subtilis* RNA polymerase holoenzyme, with the "middle" RNA polymerase from phage SPO1 infected *B. subtilis*, and with *B. subtilis* RNA polymerase core are shown in Figure 3. In the presence of 100 μM ATA, binary complexes of all three enzymes with SPO1 DNA are completely dissociated. [In a separate experiment, we satisfied ourselves that SPO1 DNA in solutions originally containing binary or ternary complexes with *B. subtilis* holoenzyme could be pelleted in the presence of 100 μM ATA (data not shown; DNA recovery was determined with DAPI as described under Materials and Methods).] Samples of the *B. subtilis* holoenzyme and of the SPO1 middle enzyme in which RNA synthesis has been allowed to initiate are not completely dissociated by ATA treatment (Figure 3a,b). Once

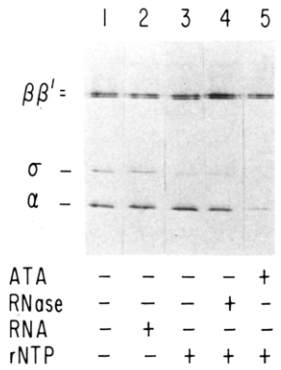

FIGURE 4: Composition of ternary transcription complexes. A mixture of 1 μg of *B. subtilis* RNA polymerase and 6 μg of SPO1 DNA was incubated as described under Materials and Methods for the formation of binary and ternary complexes. For the samples shown in lanes 1 and 2, ribonucleoside triphosphates were not added; for lanes 3–5, they were added, and RNA synthesis was allowed to proceed for 4 min. For lane 5, the "stop" mixture contained 500 μM ATA. For lane 4, 5 μg/mL RNase was present during RNA synthesis. For the sample shown in lane 2, binary complexes were first formed and then incubated with 1 μg/mL SPO1 in vivo RNA for 2 min. Samples were then prepared, centrifuged, and electrophoretically separated as described under Materials and Methods.

again, we see that ATA-treated ternary complexes contain less DNA-bound RNA polymerase β', β, and α subunits than the corresponding untreated binary complexes (compare lane 1 with lane 3 in parts a and b Figure 3, reflecting the failure of all bound RNA polymerase molecules to initiate RNA synthesis. In this respect, the *B. subtilis* RNA polymerase holoenzyme and the SPO1 middle enzyme resemble the *E. coli* holoenzyme.

It is well-known that polymerase core initiates RNA synthesis particularly poorly on intact, linear, helical DNA. The experiment shown in Figure 3c is in accord with expectation. Core enzyme–DNA binary complexes are pelleted under the conditions of this experiment (lane 1). However, ATA still strips core enzyme almost completely from its template even after incubation for 4 min with ribonucleoside triphosphates (lane 3).

B. subtilis σ and gp28 are absent from the ATA-treated ternary complexes (Figure 3a, lane 3, and Figure 3b, lane 3). One might therefore conclude that gp28 and *B. subtilis* σ are released during the elongation phase of transcription. The observation about *B. subtilis* σ has to be understood in the context of conflicting prior reports: Williamson & Doi (1979) analyzed transcribing mixtures of RNA polymerase and poly[d(A-T)] on nondenaturing gels and on glycerol gradients and concluded that σ was not released from ternary transcription complexes. Spiegelman & Whiteley (1979) analyzed transcribing mixtures of RNA polymerase and SP82 DNA which had been treated with heparin on glycerol gradients. They concluded that σ was released during the elongation phase of transcription, although approximately one-fifth of the σ still cosedimented with ternary complexes. The experiment of Figure 3 is done at lower ionic strength than either of the previous studies. One could therefore raise the question of whether or not ATA might slectively release σ from ternary transcription complexes of *B. subtilis* RNA polymerase with DNA. The experiments in the next section address that question.

Transcription Complexes in the Absence of ATA. We next analyze the composition of binary and ternary transcription complexes between *B. subtilis* RNA polymerase and SPO1 DNA which have not been treated with ATA. The important complicating factor of these experiments is that in the absence of ATA, the samples in which ternary transcription complexes have been permitted to form are not stripped of their residual binary complexes.

Table II: Loss of σ and Formation of Ternary Transcription Complexes by *B. subtilis* RNA Polymerase

	RNase added during RNA synthesis?	
	no	yes
(1) percent loss of σ with RNA synthesis (no ATA added)[a]	74 ± 2 (8)[b]	61 ± 3 (2)
(2) percent retention of α in ternary complexes[c]	43 ± 5 (7)	

[a] Measured (in the absence of ATA) as the percentage loss of σ when the B sample is converted to the corresponding T sample by adding ribonucleoside triphosphates, as described under Material and Methods. [b] Average values with average deviations; number of determinations in parentheses. [c] Measured as the percentage of ATA-resistant α subunits in T samples. Five of the determinations were made with 100 μM ATA and two with 50 μM ATA. For the latter, there was 45 ± 5% retention of α.

The results of one experiment are shown in Figure 4. Comparing the binary complexes (called the "B sample" below) in lane 1 with the sample in which RNA synthesis has been allowed to initiate (the "T sample") in lane 3, one sees that a considerable part, *but not all*, of the σ has been lost from the latter. Incomplete removal of σ from the T sample as a whole might be due to one of three causes: (1) It could be due to σ–DNA interaction; however, in separate experiments (data not shown), we found that purified *B. subtilis* σ does not bind to native SPO1 DNA sufficiently tightly to cosediment under our experimental conditions. (2) It could be a property of ternary transcription complexes. (3) It might merely signify, as already suggested above, that not all DNA-bound RNA polymerase molecules in the T sample initiate transcription.

The quantitation of binary and ternary complexes in T samples has been approached by analyzing pairs of samples, one of which has been treated with ATA to dissociate uninitiated RNA polymerase. The proportion of bound RNA polymerase molecules in T samples, which have initiated transcription, has been measured by densitometry of the subunit band in pairs of aliquots analyzed with and without ATA (such as Figure 3, lanes 3 and 4). The results of seven determinations (five separate experiments) are summarized in Table II. According to this criterion, approximately 40% of the bound RNA polymerase initiated RNA synthesis after addition of ribonucleoside triphosphates (line 2). The quantitative analysis of σ loss (e.g., Figure 3, lanes 2 and 3), however, indicates that approximately 70% of the σ was lost from DNA-bound RNA polymerase as a result of starting RNA synthesis (Table II, line 1). Two experiments show that this discrepancy (i.e., between 70% and 40%) is not entirely due to an effect of RNA chains on the release of σ from holoenzyme. First, RNA synthesis was done in the presence of enough RNase to digest more than 98% of the unincorporated RNA (Figure 3, lane 4). Only a partial reduction of the discrepancy was noted (Table II, line 1). Second, preformed binary complexes were incubated with RNA from SPO1-infected *B. subtilis* (the concentration used represents a vast excess of RNA over what is formed in vitro during 2 min of RNA synthesis), and σ was not released (Figure 4, lane 2).

We conclude that the discrepancy is primarily a property of the enzyme preparation. It could arise as a result of either of the following circumstances. (1) The enzyme preparation

Table III: Loss of gp28 and Formation of Ternary Transcription Complexes

(1) percent loss of gp28 with RNA synthesis (no ATA added)	32 ± 9 (5)
(2) percent retention of α in ternary complexes[a]	45 ± 11 (5)
(3) ratio (1)/(2)[b]	0.69 ± 0.06 (5)

[a] Resistant to 50 μM ATA; other details as specified in Table II and under Material and Methods. Two of the determinations were done with RNase present, as specified in Table II, and the ratio (1)/(2) was 0.73 ± 0.05. [b] Measured separately for each of the five determinations. Note the smaller average deviation for line 3 than for lines 1 and 2. A substantial part of the spread of values on lines 1 and 2 probably comes from experiment-to-experiment variations of RNA chain initiation.

contains a fraction of core molecules; however, the preparation of *B. subtilis* holoenzyme used for these experiments could not be stimulated to form more rapidly starting (rifampicin-resistant) ternary complexes on SPO1 DNA with added σ (data not shown). By this criterion, it is therefore σ saturated, although it might contain core molecules which can bind to DNA but cannot be stimulated by σ. (2) The enzyme preparation contains a fraction of holoenzyme molecules with a particular kind of defect: releasing σ prematurely while making a short oligonucleotide (Carpousis & Gralla, 1980) and, being unable to elongate this abortive start oligonuclotide, remaining subject to displacement by ATA. The second alternative contains some hypothetical elements but does not contradict anything that is currently known about σ release and abortive initiation. *E. coli* RNA polymerase reiteratively makes di-, tri-, and larger oligonucleotides on the *lac* UV5 promoter, even in the presence of all four ribonucleoside triphosphates (Carpousis & Gralla, 1980). No comparable experiments with RNA polymerase from Gram-positive bacteria have been reported thus far. Our own experiments show that rifampicin-poisoned RNA polymerase complexes, which make dinucleotides in the presence of ribonucleoside triphosphates (McClure & Cech, 1978; McClure et al., 1978), remain sensitive to displacement by ATA (data not shown). We have not further investigated the properties of inactive RNA polymerase molecules.

The results of a series of similar experiments with E.gp28 are presented in Table III. The release of gp28 correlates with the formation of ternary complexes. However, there is consistently less gp28 release than initiation. This deficit implies that gp28 has a somewhat greater affinity than σ does for some component of the ternary transcription complex. The following experiments appear to eliminate RNA and DNA as responsible for this affinity: (1) Including RNase A in the T sample did not result in increased release of gp28. (2) Gp28 does not bind to native SPO1 DNA sufficiently tightly to cosediment under our experimental conditions (data not shown). (3) We also prepared SPO1 DNA which had been digested with exonuclease III to expose, on the average, approximately 12 kilobases of single-strand termini. This DNA (3 μg) was mixed with gp28 (approximately 30 ng) in the standard incubation medium and sedimented. Once again, gp28 did not bind sufficiently tightly to cosediment with the DNA (data not shown). One is forced to conclude, by this process of elimination, that the gp28 remaining with the ternary complex is due to a residual affinity for elongating RNA polymerase.

Discussion

The experiments described here prove that the RNA polymerase core enzyme of *B. subtilis* changes its interaction with two subunis, σ and gp28, during transcription. The comparable properties of these two initiation specificity determining subunits are in keeping with the notion that they are functional analogues (Talkington & Pero, 1979), adapting the same enzymatic machinery to alternative but isomorphous signals. These results do not prove that there are σ and gp28 cycles in vivo, but they make that model as likely for *B. subtilis* as for *E. coli*. Of course, the notion of a σ cycle implies that the cell contains an excess of RNA polymerase core over specificity factor; the experimental evidence on this point is lacking both for uninfected and for SPO1-infected *B. subtilis*.

It has been relatively simple to show that the relatively ATA-resistant ternary transcription complexes formed by *B. subtilis* RNA polymerase holoenzyme and by the phage SPO1 modified middle enzyme lack the σ and gp28 subunits, respectively, and that the latter are therefore not required for elongation of RNA chains. However, the possibility that ATA is responsible for stripping these subunits from ternary complexes cannot, a priori, be excluded. For that reason, ternary transcription complexes have also been examined in the absence of ATA. The problem posed by this analysis is that even under conditions that should favor the formation of open promoter complexes, by no means all the DNA-bound RNA polymerase molecules initiate RNA synthesis. The preparations of enzyme used for these experiments do contain a sizable fraction of inactive molecules (Chamberlin et al., 1979), perhaps including some that are competent to bind to DNA but unable to initiate efficiently. Moreover, electron microscopic examination of binary complexes under comparable conditions indicates a certain fraction of RNA polymerase molecules which is not bound at promoter sites (Romeo et al., 1981). Possibly, the sizable fraction of noninitiating RNA polymerase molecules is the source of the disagreement between our results with *B. subtilis* holoenzyme and a prior report (Williamson & Doi, 1979), involving experiments in which relatively large quantities of RNA polymerase were mixed with poly[d(A-T)] and extensively dialyzed before initiating RNA synthesis. The qualitative agreement between our results and those of Spiegelman & Whiteley (1979), who also utilized bacteriophage DNA in their assay, might point to the possibility of free σ binding to poly[d(A-T)]. Kudo et al. (1981) have, in fact, presented data which show that *B. subtilis* σ binds to DNA, in particular, to supercoiled plasmid DNA. This is in contrast with our results with linear phage DNA, possibly due to the supercoiling or to the much higher σ-to-DNA ratio used by Kudo et al. (1981). In either case, under our conditions, where that possible artifact has been eliminated, the results are clear.

Gp28 apparently has a greater affinity for RNA polymerase core in ternary transcription complexes than σ does. This is indicated by the incomplete separation of gp28 from ternary complexes during centrifugation (Table III). Differences in the affinity of specificity factors for elongating core RNA polymerase could have implications for cells with multiple factors. The subunit which binds with greater affinity to ternary complexes, gp28 in this case, would be at an advantage for directing a particular molecule of elongating core enzyme in the next round of transcription. This would effectively sequester some RNA polymerase core on only one type of promoter, even in cells containing multiple specificity factors, and would provide a means by which factors present as only minor components could be effective while sharing a common cytoplasm with a functioning major specificity factor. It should be noted that this model relies on the existence of an extremely small pool of free RNA polymerase core, a condition which

may well exist in actively growing and in phage-infected cells. In the SPO1 phage infected cell, accessory proteins, which remain to be identified, may be required for turning off early transcription.

Although it is a subsidiary matter, we have noted that some of the impurities in our RNA polymerase preparations have certain common properties. They bind to DNA along with RNA polymerase but are stripped off by ATA. We do not have these impurities as purified proteins, that is, without RNA polymerase. We cannot therefore directly determine whether these contaminants are RNA polymerase binding proteins or ATA-sensitive DNA-binding proteins. However, if they are the latter, they must all share certain characteristic chromatographic properties with RNA polymerase, such as binding both to cation and anion exchangers. By way of contrast, the σ and gp28 subunits do not, by themselves, bind to helical or single-stranded SPO1 DNA sufficiently strongly to withstand centrifugation.

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