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Delta Factor can Displace Sigma Factor from *Bacillus subtilis* RNA Polymerase Holoenzyme and Regulate Its Initiation Activity

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Summary. A protein with a molecular weight of 21,000 daltons is found associated with a fraction of *Bacillus subtilis* RNA polymerase core. This protein (δ) does not react with antibody made against sigma factor and has a peptide map which is significantly different from sigma factor. At ratios of 2:1 to 4:1 (δ:holoenzyme) the δ displaces sigma factor completely from the core and associates in a 1:1 ratio with core to form δ-core. Under the same incubation conditions sigma factor at a ratio of 10:1 (sigma factor:δ-core) does not displace δ from the δ-core. The δ-core has much less activity as compared to holoenzyme on various DNA templates. However, sigma factor does stimulate the activity of δ-core enzyme under conditions of RNA synthesis. These observations and the results of others suggest that δ-core enzyme binds initially to specific DNA sites followed by δ release from the core-DNA complex and that the sigma factor binds to the core-DNA complex to initiate RNA synthesis. Thus both δ and sigma factors are required in a sequential fashion for specific transcription to occur in *B. subtilis*.

Introduction

Several investigators have reported the presence of a δ protein, also called 21K, with a molecular weight of 21,000 daltons associated with the RNA polymerase core of *Bacillus subtilis* (Halling et al., 1977; Plevani et al., 1977; Pero et al., 1975; Spiegelman et al., 1978; Tjian et al., 1977). As yet a definitive function for δ has not been identified, although its effect on increasing SP01 and SP82 phage middle gene transcription (Pero et al., 1975; Spiegelman et al., 1978) and on decreasing poly [d(AT)] transcription (Tjian et al., 1977) have been noted. The results presented by Halling et al. (1977) and Plevani et al. (1977) have shown that the δ-core enzyme elutes from the DNA-cellulose column at an earlier position than holoenzyme and contains one δ per core.

We have investigated the chemical and functional relationship between δ and sigma factor in order to understand the role of δ in transcription. Our results indicate that sigma and δ are products of different genes since they are not immunologically related nor do they have similar peptide maps. Furthermore by in vitro experiments we have found that δ can displace sigma factor completely from holoenzyme and bind to the core in a 1:1 ratio to form δ-core. The addition of excess sigma factor to δ-core enzyme did not result in displacement of δ by sigma factor. The pure δ-core enzyme has little activity on various DNA templates. However, the addition of sigma factor to δ-core enzyme stimulated its activity on DNA templates. These results suggest that sigma factor may interact with the δ-core only when it is associated with the DNA template.

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Materials and Methods

Bacterial Strain and Media. Bacillus subtilis 168 wild type was used for all the experiments. Cells were grown in a tryptose-yeast extract medium (Halling et al., 1977) at 37° C with vigorous aeration to a density of 2×10^9 cells/ml.

RNA Polymerase Purification. The method of Halling et al. (1977) was used to purify RNA polymerase through the DNA-cellulose column chromatography step. The δ-core eluted at lower KCl concentrations than the holoenzyme and these two enzyme fractions were separated at this point (Halling et al., 1977).

Buffers. Buffer D contained 20 mM Tris-HCl, pH 7.9, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), 1 mM phenyl-methylsulfonylfluoride (PMSF), and 20% glycerol. Buffer E contained 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 1 mM PMSF, and 10% glycerol. Buffer

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C (competition buffer) contained 20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 15% glycerol, and 0.12 M KCl. Glycerol gradient buffers were made with Buffer C containing glycerol from 20% to 40%.

Sigma, δ and Core Purification. After DNA cellulose chromatography the enzyme (either pooled enzyme, holo-, or δ-core) (10–30 mg) was dialyzed to 0.1 M KCl in Buffer D, loaded onto a DEAE-cellulose column (0.9 × 6.0 cm), then eluted with buffer D, 0.6 M KCl. This procedure concentrated the enzyme. The enzyme was then dialyzed for at least 24 hr *versus* Buffer E, 0.02 M KCl and loaded onto a phosphocellulose column (2.6 × 17 cm) equilibrated with the same buffer. The column was washed with Buffer E, 0.02 M KCl. Sigma and δ were both eluted in the wash. Core enzyme was eluted with Buffer E, 0.5 M KCl. Traces of core and other minor contaminants were removed by application of the phosphocellulose column flow-through fractions to a DNA-cellulose column (1.6 × 15 cm). Sigma and δ appeared in the DNA-cellulose column flow-through fractions. This fraction was then applied to a DEAE-cellulose column (0.9 × 3.0 cm) and eluted with a salt gradient of 0.1 M to 0.5 M KCl (30 × 30 ml). Sigma is eluted at about 0.18 M KCl and δ at about 0.25 M KCl. The two proteins appear pure by SDS gel electrophoresis (Fig. 1).

RNA Polymerase Assay. The reaction mixture contained in a total volume of 0.125 ml: 40 mM Tris, 10 mM $MgCl_2$, 0.4 mM DTT, 0.2 mM each of ATP, GTP, and CTP, 0.2 mM UTP (^{3}H-UTP specific activity 2.5×10^4 cpm per nmole); KCl concentration was 0.1 M for φe DNA (2.5 μg) or for φ29 DNA (2 μg) and 0.03 M for *B. subtilis* DNA (1 μg). Incubation was for 10 min at 37° C and reaction was terminated by addition of 0.125 ml of 10% TCA at 0° C.

Peptide Mapping Technique. The protease digest technique of Cleveland et al. (1977) was used. Protein samples (9 μg) were brought to 0.5% SDS and heated to 100° C for 3 min. After cooling, the protease was added and the mixture was incubated at 37° C for 30 min. The SDS-urea-polyacrylamide gel sample application buffer of Wu and Bruening (1971) was added with enough mercaptoethanol to bring the final concentration to 10%. Samples were heated to 100° C for 3 min before application to 12% acrylamide-SDS-urea slab gels.

Cyanogen bromide cleavage as adapted from Gross (1967) was performed as follows: the protein samples were freeze-dried, then dissolved in 50 microliters of 70% formic acid. One crystal of cyanogen bromide was added to the sample and the mixture was incubated 48 hr at room temperature, freeze-dried, then dissolved in water; the peptides were separated as described for the protease digests.

Antibody Production. Antibody against sigma factor was produced by injecting rabbits with 100 micrograms of sigma factor in Freund's complete adjuvent for three times at two week intervals. Rabbits were injected in the popliteal lymph nodes and were bled by cutting the ear.

Competition Studies. Approximately 0.3 mg of the RNA polymerase species to be tested was combined with the appropriate amount of competitor factor (either σ or δ) and the volume was brought to 0.5 ml with Buffer C. The mixture was dialyzed overnight *versus* 500 ml of Buffer C. Linear gradients of 20% to 40% glycerol (3.7 ml total volume) were prepared in 7/16 × 2–3/8 inch polyallomer tubes. The dialyzed sample was layered on the gradient and centrifuged for 26 hr at 157,000 × g in a Beckman type 56 swinging bucket rotor. Gradients were collected by piercing a hole in the bottom of the tube. 0.5 ml fractions were collected and run on a 10% polyacrylamide slab gel.

Protein Assay. Protein concentration was determined by the method of Sedmak and Grossberg (1977).

Results

Comparison of the δ Protein and Sigma Factor. The δ protein as reported previously was found associated with core enzyme (δ-core) which eluted in front of the holoenzyme from a DNA-cellulose column (Halling et al., 1977; Plevani et al., 1977). No δ was found in the holoenzyme fractions, nor was sigma factor found associated with δ-core (Halling et al., 1977; Plevani et al., 1977). The proteins ω_1 (11,000 daltons) and ω_2 (9,500 daltons) which have been reported to be associated with RNA polymerase (Duffy and Geiduschek, 1975) were also found. The δ-core and holoenzyme fractions both contained ω_1 in a ratio of about 1:1, but ω_2 was found associated mainly with the holoenzyme fraction (Table 1). The δ and sigma factor were purified as described in the Methods and their SDS-urea-polyacrylamide gel electrophoresis patterns indicated that they were pure (Fig. 1). The sigma factor has a molecular weight of 54,000 daltons while the δ protein has a molecular weight of 21,000 daltons (Pero et al., 1975; Spiegelman et al., 1978).

The immunological relationship between sigma and δ was tested, since it was possible that δ was a fragment of sigma factor. A double diffusion analysis with the Ouchterlony plate revealed that anti-sigma antibody did not react with δ protein (Fig. 2). This was good evidence that the two polypeptides were different in amino acid sequence.

The two proteins were compared further by analysis of their peptide patterns by the method of Cleveland et al. (1977) and by the CNBr cleavage method. The cleavage patterns were very reproducible among several experiments. Results by both of these methods revealed a significant difference in the peptide map patterns between sigma and δ (Fig. 3). Thus δ differed from sigma factor in molecular weight, antigenic properties, and peptide map.

Displacement of Sigma Factor from Holoenzyme by δ. To determine whether δ would compete with sigma factor for the RNA polymerase core, several competition experiments were performed. In the first experiments, pure holoenzyme was mixed with varying amounts of δ protein and incubated for 24 h at 4° C in Buffer C (competition buffer) (see Methods). The mixture was then layered on a 20–40% glycerol gradient and centrifuged for 26 h at 157,000 × g in a Beckman SW56 rotor. Fractions were collected from the glycerol gradient and each fraction was analyzed for the presence of holoenzyme subunits (α, β, β′,

Table 1. Stoichiometry of RNA polymerase subunits after incubation with δ and σ factors and glycerol gradient centrifugation[a]

	Incubation Mixtures	σ/α_2	δ/α_2	ω_1/α_2	ω_2/α_2
a.	δ-core enzyme + σ (4×)[b]	0.02	1.01	1.01	0.22
b.	δ-core enzyme + σ (10×)[b]	0.04	1.00	0.74	–
c.	Holoenzyme + δ (4×)[b]	0.04	1.12	0.98	0.92
d.	Holoenzyme + δ (2×)[b]	0	1.28	1.08	–
e.	Core + δ (1×)[b] + σ (1×)[b]	0.01	0.96	1.07	0.46
f.	Core + σ (2×7)[b]	0.57	0	1.18	–
g.	δ-core enzyme	0.03	1.06	1.08	0.27
h.	Holoenzyme	0.68	0.05	1.02	0.72
i.	Holoenzyme[c]	0.74	0	1.10	0.72
j.	δ-core enzyme[c]	0.03	1.06	1.25	0.26

[a] These results are the averages of two independent values obtained from densitometric tracings of glycerol gradient enzyme fractions which were analyzed by SDS polyacrylamide gels (see Methods). Gels were stained with Coomassie Blue and scanned at 600 nm. The ratio of the factors to the core was based on the α_2 content of the core

[b] The values in parentheses are the molar excess of factor added to holoenzyme, δ-core enzyme, or the core enzyme

[c] This is the composition of the enzymes prior to glycerol gradient centrifugation. The enzyme fractions were obtained from a DNA-cellulose column

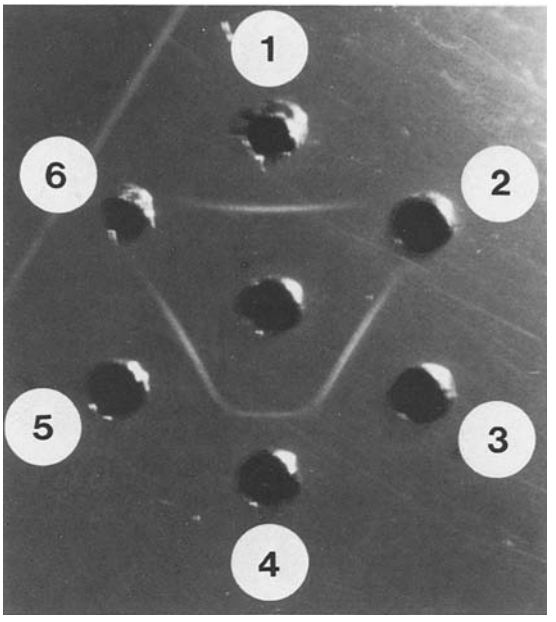

Fig. 2. A double diffusion analysis of δ factor with anti-σ antibody. Center well contained anti-σ serum. Wells 1, 3, and 5 contained σ (0.11 mg/ml); wells 2 and 6 contained δ (0.05 mg/ml); well 4 contained holoenzyme (0.5 mg/ml). Each well contained 12 µl of each sample

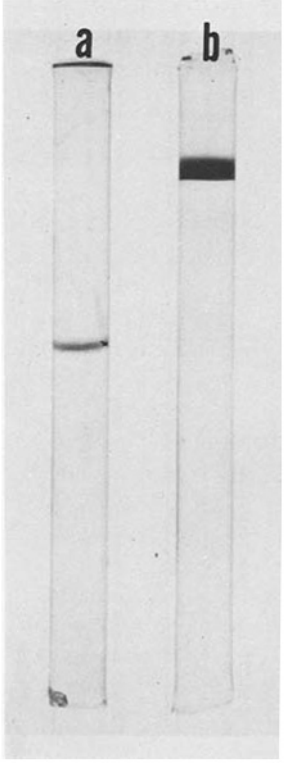

Fig. 1a and b. SDS-urea-12% polyacrylamide gel electrophoresis patterns of purified **a** δ factor and **b** σ factor

and σ) and δ by slab gel electrophoresis. The results in Figure 4 and Table 1, lines c and d, illustrate that the δ protein completely replaced sigma factor from the holoenzyme when it was present at as low as a 1:1 ratio with the holoenzyme in the competition mixture and sedimented as the δ-core through the glycerol gradient. The control experiments indicated that holoenzyme itself was stable under these experimental conditions (Table 1, lines g and h). The stoichiometry of the displacement reaction is summarized in Table 1. In the reciprocal experiments when σ factor was added at a 10-fold excess to δ-core, very little or no displacement of δ occurred (Table 1, lines a and b) and there was no binding of σ to the core. These results indicate that δ has a much higher affinity than sigma factor for core under these incubation conditions and that the δ forms a tight complex with core, since the δ-core complex can be sedimented through a glycerol gradient. When core was mixed with equimolar amounts of δ and sigma factor, the resulting complex was comprised essentially of δ-core enzyme (Table 1, line d). It is also worthy to note that while δ-core obtained from the DNA-cellulose column fractions contained little ω_2, δ did not displace ω_2 from the holoenzyme.

Effect of δ on in vitro Transcription. The activity of holoenzyme and δ-core enzyme was tested on various

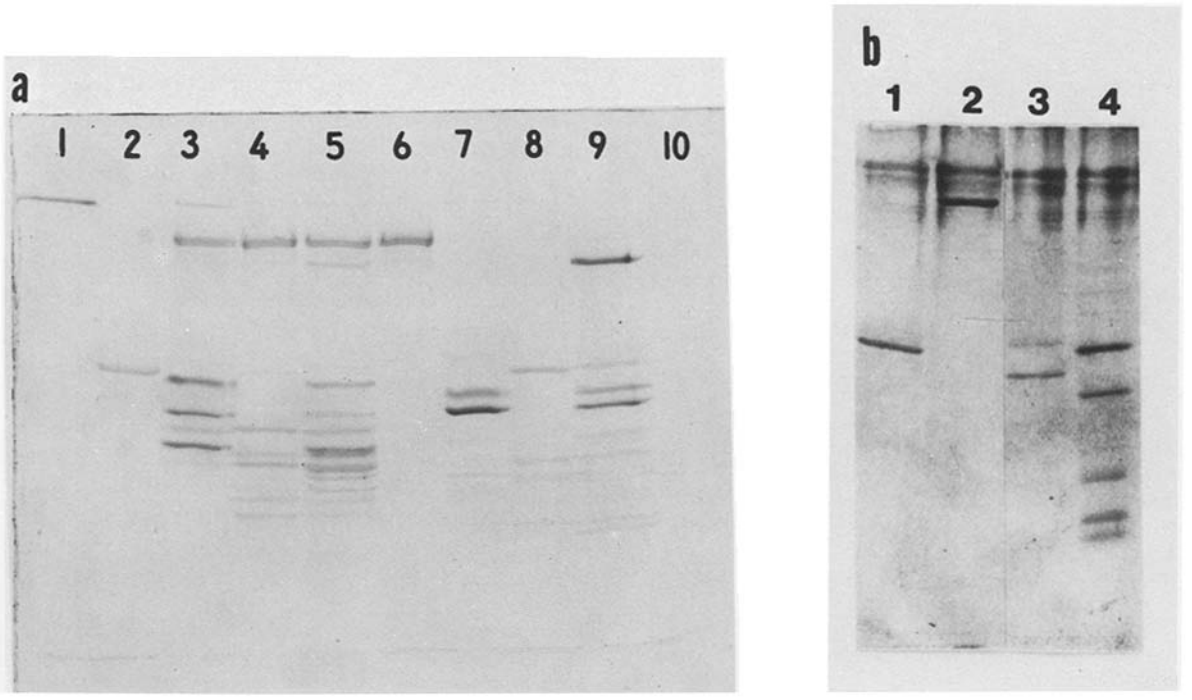

Fig. 3. a Protease cleavage patterns of σ and δ factors. Cleavage and electrophoresis were carried out as described in Methods. 1) 3 μg σ; 2) 3 μg δ; 3) 9 μg σ+3 μg *S. aureus* V8 protease; 4) 9 μg δ+3 μg V8 protease; 5) 9 μg σ+9 μg δ+6 μg V8 protease; 6) μg V8 protease; 7) 9 μg σ+0.15 μg subtilisin; 8) 9 μg δ+0.15 μg subtilisin; 9) 9 μg σ+9 μg δ+0.3 μg subtilisin; 10) 0.3 μg subtilisin. **b** CNBr cleavage patterns of σ and δ factors. 1) 3 μg δ; 2) 5 μg σ; 3) CNBr cleaved δ (4 μg); 4) CNBr cleaved σ (15 μg)

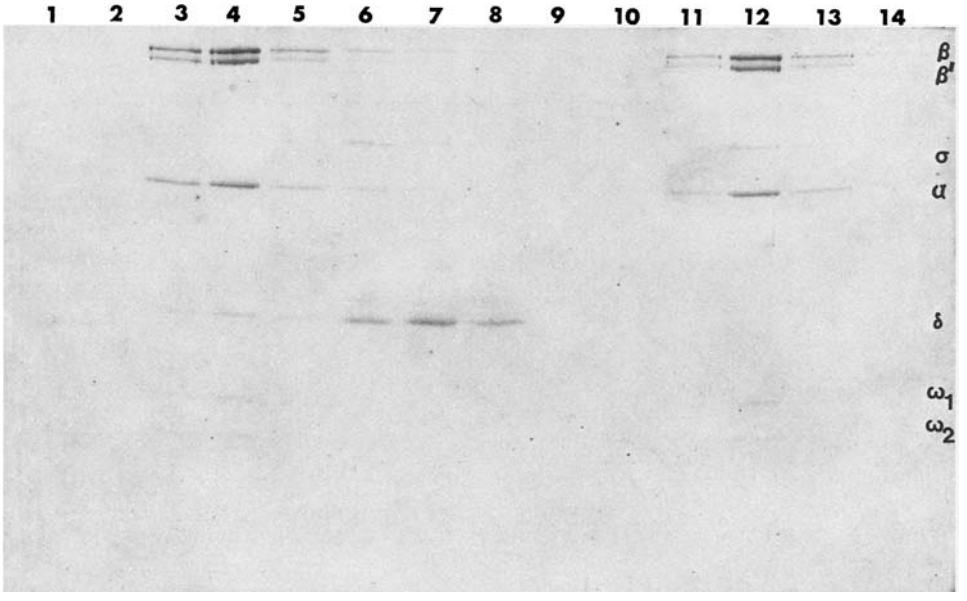

Fig. 4. SDS-urea-12% polyacrylamide gel electrophoresis patterns of fractions after glycerol gradient centrifugation. Wells 1–8 show glycerol gradient fractions in which 4 fold excess δ was added to holoenzyme prior to centrifugation. Well 1 is the bottom and well 8 is the top of the gradient. Wells 9–14 show control in which holoenzyme was centrifuged. This is the lower 6 fractions of the gradient. No bands were observed in the upper two fractions

Table 2. Specific activity of holoenzyme, δ-core enzyme, and core enzyme on various DNA templates[a]

Enzyme	φe DNA	φ29 DNA	*B. subtilis* DNA
Holoenzyme	212	357	108
δ-core enzyme	17	6.6	0.4
Core enzyme	12	16.5	6.6

[a] nmoles UMP incorporated/10 min/mg protein. Each number is the average of the results from six independent reactions

Table 3. Stimulation of δ-core by sigma factor and the effect of δ on holoenzyme

Enzyme	Specific activity
δ-core	0.8
δ-core + σ (2×)[a]	14.5
Holoenzyme	96.6
Holoenzyme + δ (1×)	11.8
Holoenzyme + δ (3×)	13.3

[a] The numbers in parentheses indicate the molar ratio of the added factor to the δ-core or holoenzyme. The template was *B. subtilis* DNA (1 μg)

templates. The results in Table 2 illustrate that holoenzyme has much higher specific activity on phage and *B. subtilis* DNA than either δ-core enzyme or core enzyme. The holoenzyme activity on φe DNA, φ29 DNA, and *B. subtilis* DNA was 12.5, 54, and 270 times more active than δ-core enzyme activity, respectively. With respect to core enzyme activity, δ-core enzyme was 1.4, 0.4 and 0.06 times as active on the three templates, respectively. Generally the δ-core enzyme had very low activity relative to both holoenzyme and core enzyme.

The effect of sigma factor on δ-core enzyme was also tested with *B. subtilis* DNA as template. The results in Table 3 show that the presence of sigma factor stimulated the activity of δ-core enzyme by 18-fold and to a specific activity of 14.5. Of additional significance is the fact that the addition of δ to holoenzyme reduced the specific activity of holoenzyme from about 97 to about 12, or to the same specific activity as when sigma factor was added to δ-core. These results indicated that sigma in an RNA synthesizing system could interact with either the δ-core enzyme or the core enzyme and carry out RNA synthesis at a much higher rate than δ-core enzyme alone.

The effect of σ factor however varied with the template used. The results of σ factor on transcription when φe DNA was the template are summarized in Table 4. When δ factor was added to holoenzyme, no change in activity was noted. When σ factor was added to δ-core, a five-fold stimulation in activity

Table 4. Activity of holoenzyme, δ-core enzyme and core enzyme on φe DNA

	Enzyme	Specific activity[a]
a.	Holoenzyme	195.0
b.	Holoenzyme + δ (6×)	193.0
c.	δ-core	29.6
d.	δ-core + σ (1.6×)	89.2

[a] nmoles ^{3}H-UMP incorporated/10 min/mg protein. The figures in parentheses are the molar ratios of the factors to the enzyme

was observed. The lower degree of activation of the δ-core by σ factor (i.e., 89 instead of 193) is possibly due to the lack of core saturation by σ. In any case it appears that the promoters on φe DNA are strong enough to bind both holoenzyme and δ-core enzyme plus σ with equal efficiency (Table 4, lines a and b).

Discussion

The results clearly indicate that δ can effectively displace sigma factor from the holoenzyme in the competition buffer. This displacement must happen rapidly, since the addition of δ to an RNA synthesis reaction mixture containing holoenzyme also had a significant effect (see Table 3). Therefore, whenever core enzyme or holoenzyme is in the presence of δ and sigma factor, the core enzyme will be converted to the δ-core enzyme.

However the stimulatory effect of sigma factor on the activity of δ-core in an RNA synthesis reaction mixture indicated that sigma could still interact either with the δ-core enzyme or DNA-bound core enzyme which had lost δ. The data of Spiegelman et al. (1978) with δ-core enzyme indicated that δ fell off of the core when a mixture of δ-core and holoenzyme interacted with DNA. Thus our interpretation of the results of sigma stimulation of δ-core activity is that initially δ-core enzyme binds to the DNA; upon binding to a promoter region the δ falls off of the enzyme-DNA complex and now sigma factor is able to bind to core and initiate RNA synthesis. In our model the δ polypeptide (DNA specificity factor) determines the binding of the core enzyme to specific DNA sites and sigma factor (initiation factor) stimulates the initiation of RNA synthesis. Thus in *B. subtilis* two separate polypeptides may be functioning as the single polypeptide sigma factor in *E. coli*. Since the *E. coli* sigma factor has a molecular weight of 90,000 daltons (Burgess, 1969) and the δ and sigma factors of *B. subtilis* together weigh about 75,000 daltons, it is possible that the single polypeptide *E. coli* sigma factor

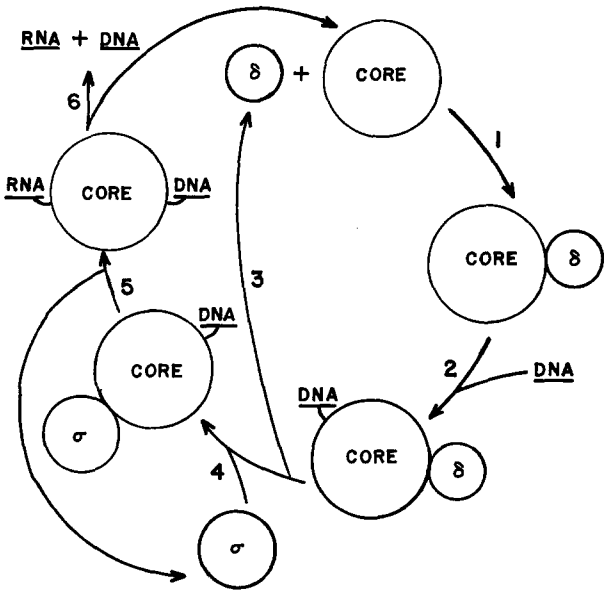

Fig. 5. A model of δ and σ factor interaction with RNA polymerase core during binding to DNA and initiation of RNA synthesis. Step 1, δ binds to core forming δ-core enzyme; step 2, δ-core binds to promoter site of DNA resulting in a DNA-δ-Core complex; step 3, δ factor is released from the DNA-δ-core complex allowing σ factor to form the σ-core-DNA complex (step 4); step 5, after initiation σ factor is released; step 6, elongation occurs with eventual release of RNA, DNA, and core enzyme

has the functional information present in both the *B. subtilis* δ and sigma factors.

There is evidence that δ is involved in increasing the specificity of phage DNA transcription. Plevani et al. (1977) showed that transcription from SPP1 DNA was more asymmetric with δ core than with core. Spiegelman et al. (1978) showed that δ increased the translatability of SP82 DNA transcripts with both holoenzyme and phage modified enzyme. Previous studies by Pero et al. (1975) suggested that the δ factor was increasing the specificity of gene transcription with SP01 phage-modified RNA polymerase. It is thus possible that the presence of δ factor with σ-enzyme results in a more limited recognition of specific promoters. Thus the high activity of holoenzyme on *B. subtilis* DNA (Table 3) relative to the activity of holoenzyme plus δ factor suggests that holoenzyme alone may be transcribing from more sites than the δ-core-σ enzyme. These sites may be nonspecific sites such as nicks or single stranded regions of the sheared *B. subtilis* template which allow binding and initiation by holoenzyme in the absence of δ factor. The decreased activity of the δ-core-σ enzyme on poly [d(AT)] observed by Tjian et al. (1977) may have been caused by the absence of a true promoter on this synthetic template. On the other hand both Tjian et al.'s (1977) and our results (Table 4) with ϕe DNA indicate that the promoter sites on this phage DNA have strong affinity for both holoenzyme and δ-core-σ enzyme, since the addition of δ factor to the holoenzyme did not change its transcription activity on this template. A role for δ in causing reduced binding to nonspecific sites, one of the functions of σ in *E. coli* (Chamberlin, 1974), is quite likely. Spiegelman et al. (1978) have suggested this in DNA binding studies using SP82 DNA. This could also be reflected in the early elution of δ-core from the DNA cellulose column.

Since the relative ratio in vivo of δ factor to σ factor is still unknown, it is difficult to decide whether all core enzyme proceeds through a δ-core complex prior to RNA synthesis or whether some core can interact directly with σ factor to form holoenzyme which can bind and initiate proper RNA synthesis. Further studies are necessary to determine whether the δ-core is mandatory for in vivo transcription.

A working model of the interaction of δ and σ factors with RNA polymerase core is presented in Figure 5. In this model δ first combines with the core to form δ-core which binds to the promoter site on DNA. Upon associating with the promoter, the conformation of the δ-core complex is altered resulting in the release of δ from the δ-core-DNA complex. At this point σ factor binds to the core and after initiation of RNA synthesis, the sigma factor dissociates from the core (Travers and Burgess, 1969). Thus δ and σ factors act sequentially and the reactions result in a δ cycle and a σ cycle.

Several events can be predicted from this model. (a) Bacterial species have been found which contain either 90,000 or 55,000 dalton sigma factors (Nakamura et al., 1977). Those species which have been shown to contain a 55,000 dalton σ factor may also contain a δ factor. (b) Two types of RNA synthesis initiation mutants will be identified: mutants with enzymes which cannot bind DNA and another type which can bind but not initiate RNA synthesis. In *E. coli* the mutations giving rise to these phenotypes will be tightly linked, i.e., in the σ gene (Nakamura et al., 1977; Harris et al., 1977), since the two functions are fused into one polypeptide. In *B. subtilis* the mutations for the two phenotypes will occur in separate genes, since the functions appear in two separate polypeptides; another possibility, however, is that the δ and σ factors arise from a single polypeptide which is rapidly cleaved by some proteolytic activity. The occurrence of this type of cleavage during purification of RNA polymerase from *B. subtilis* is unlikely since the use of protease inhibitors and the hemoglobin-Sepharose protease affinity column (Nakayama et al., 1977) removes protease activity very efficiently from crude extracts. Other evidence has been

presented for fused and separated genes in *E. coli* and *B. subtilis* for similar enzymatic functions (Kane et al., 1972; Grieshaber and Bauerle, 1972; Jackson and Yanofsky, 1974). (c) The occurrence of σ' factor in late logarithmic and stationary phase cultures of *E. coli* (Fukuda et al., 1974) and *B. subtilis* (Halling and Doi, submitted for publication, 1977) and of δ^1 and δ^2 polypeptides in sporulating *B. subtilis* cells (Fukuda and Doi, 1977; Linn et al., 1975) suggest that these polypeptides may be related to vegetative cell σ and δ factors. The role of these factors are currently being investigated in differential gene transcription during bacterial sporulation (Doi, 1977).

The *B. subtilis* RNA polymerase which differs significantly in structure and subunit function from the *E. coli* enzyme should serve as an extremely suitable system for dissecting the mechanism of promoter recognition and initiation of RNA synthesis.

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