The effect of the delta subunit on the interaction of *Bacillus subtilis* RNA polymerase with bases in a SP82 early gene promoter

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ABSTRACT

We have examined the effect of the delta subunit on the interaction of the *Bacillus subtilis* RNA polymerase with an early gene promoter of phage SP82. Methylation by dimethyl sulfate, used to probe close approaches of polymerase to purines, revealed that noninitiated complexes formed by holo-enzyme (core-sigma-delta) had significantly fewer contacts than complexes formed by core-sigma. The presence or absence of delta had little or no effect on close approaches to purines in initiated complexes. DNAase I footprinting indicated that core-sigma was bound to the same region regardless of whether delta, initiating nucleotides, or both, were present. These data support the conclusion that delta acts prior to initiation to enhance promoter selectivity by limiting the number of possible interactions that the polymerase can make with DNA.

INTRODUCTION

A variety of techniques have been used to probe interactions between *Escherichia coli* RNA polymerase and promoters (1). Most of the enzyme-DNA "contacts" detected in these studies are clustered around two areas of recognized conserved base sequence, the -10 and -35 regions. While the primary use of these techniques has been to help define the interaction of *E. coli* polymerase with various promoters, they also offer a means of comparing the complexes formed by different forms of polymerase with a characterized promoter.

Although the basic subunit structure of the *Bacillus subtilis* RNA polymerase is similar to that of *E. coli*, the predominant *B. subtilis* polymerase has a smaller sigma subunit ($M_r = 55,000$) and contains an additional subunit, delta ($M_r = 21,000$). It is possible to isolate two transcriptionally active forms of the major *B. subtilis* polymerase, the holo-enzyme (core-sigma-delta) and a form lacking delta (core-sigma). A number of investigations (2,3) have shown that transcriptional specificity is influenced by delta -- i.e., at low ionic strength, core-sigma displays poor

specificity. We found that removal of delta resulted in the formation of stable complexes between core-sigma and nonpromoter sites on the DNA and that RNA synthesis was initiated from many of these sites if ribonucleoside triphosphates were added (4). The observation that delta eliminates the formation of complexes at nonpromoter sites suggests that this peptide may influence the interaction between core-sigma and DNA. This possibility has been examined by analyzing noninitiated and initiated polymerase-promoter complexes using methylation (5) to detect close approaches to purines and "footprinting" to determine protection from DNAase I (6).

METHODS

Procedures using previously described methods. *B. subtilis* RNA polymerase (3), delta (3) and core-sigma (4) were purified as described. Promoter-bearing restriction fragments were identified by measuring the formation of complexes between polymerase and restriction fragments of SP82 DNA under noninitiating and initiating conditions using the filter-binding assay of Jones *et al.* (7) with some modifications (4). Methods have been described for the synthesis of RNAs in the presence of α -³²P-UTP (8) and their hybridization to the separated strands of SP82 DNA (9) or restriction fragments of SP82 DNA immobilized on nitrocellulose membrane filters (10). All restriction digestions were performed in buffers recommended by the manufacturer, New England Biolabs. SP82 DNA was digested with HaeIII and the two smallest fragments, Hae D and E, were separated by sedimentation through sucrose gradients (11). Protein concentrations were determined according to Bradford (12).

Preparation of purified DNA fragments. The combined Hae D and E fragments (1 mg/ml DNA) were digested with Sau3AI and preparatively electrophoresed through a 4% polyacrylamide (30:1 acrylamide:bisacrylamide) gel in 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA (TBE). A 250 bp band was identified after ethidium bromide staining, cut from the gel and recovered by diffusion from the gel, followed by chromatography on DE-52 cellulose and ethanol precipitation (13).

End-labeling of DNA fragments. The purified DNA fragment was dephosphorylated with bacterial alkaline phosphatase (Bethesda Research Labs) and 5' end-labeled with polynucleotide kinase (New England Nuclear) and γ -³²P-ATP ($\sim$ 3000 Ci/mmol, New England Nuclear) as described by Maxam and Gilbert (14). Alternatively, the 3' end was labeled by "fill-in" of the 5' extension using 5-10 pmoles of DNA and 2 units of *E. coli* DNA polymerase

I large fragment (New England Nuclear) in 25 μ l of 40 mM Tris, pH 7.4, 6 mM 2-mercaptoethanol, 6 mM $MgCl_2$, 100 μ M dGTP and 0.3 μ M α - ^{32}P -dATP (New England Nuclear, ~ 3000 Ci/mmol) at 20°C for 30 min. The two labeled ends of the Sau3A 250 bp fragment were separated by polyacrylamide gel electrophoresis after cleavage with DdeI (the site of the cleavage is shown in Fig. 1). The larger fragment (230 bp in length), designated Sau3A-Dde 230, was used for all methylation and footprinting.

Sequence determinations. The DNA base sequence determination was as described by Maxam and Gilbert (14) using reactions R5, R6, R7 and R8 except R6 was performed at 37°C for 10 $\pm$ 5 min. Contrary to an earlier report (15), the formic acid depurination (reaction R6) was not inhibited by the presence of the substituted base, 5-hydroxymethyluracil.

RNA was labeled at the 5' end by transcribing the Sau3A 250 bp fragment in a reaction mixture containing 1 μ g of *B. subtilis* RNA polymerase, 40 mM Tris, pH 7.9, 20 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-mercaptoethanol with 200 μ M UTP, CTP and GTP and 70 μ M γ - ^{32}P -ATP (~ 3000 Ci/mmol, New England Nuclear) in a 5 μ l reaction volume. The RNA was purified by electrophoresis through a 10% polyacrylamide-7 M urea TBE gel, located by autoradiography, eluted as described for DNA by Maxam and Gilbert (14) and ethanol precipitated two times. RNA purines were determined using the limited cleavage reactions of RNAase T₁ and RNAase U₂ according to Donis-Keller *et al.* (16).

Methylation protection. The Sau3A-Dde 230 fragment, labeled on a single 3' or 5' end was incubated with 10 μ g of *B. subtilis* core-sigma plus 1 μ g of delta or the equivalent volume of "delta storage buffer" (0.1 M NaCl, 10 mM Tris, pH 7.9, 1 mM EDTA and 30% glycerol) in 100 μ l of buffer B (5) at 37°C. Complexes were allowed to form for 10 min then 5 μ g of single-stranded M13 DNA was added to dissociate loosely bound polymerase molecules and prevent the rebinding of free polymerase. After 1 min, samples were methylated with dimethyl sulfate (DMS) for 30 sec at 37°C and filtered as described by Johnsrud (5). DNA was eluted from filters with gel elution buffer (14) at 60°C for 10 min and ethanol precipitated after addition of 10 μ g of yeast tRNA (Boehringer-Mannheim). Hydrolysis at methylated purines was done in 1.5 ml snap-cap tubes in 35 μ l of 5 mM sodium phosphate, pH 7.2, for 15 min at 90°C, followed by addition of 2 μ l of 1.2 M NaOH, and further incubation for 15 min at 90°C. The samples were ethanol precipitated and resuspended in 2-5 μ l of urea-dye mix (5 M urea, 0.1% bromophenyl blue, 0.1% xylene cyanol FF) for gel electrophoresis. When the polymerase-DNA

interaction was studied under conditions of initiation, ATP and CTP were added to a final concentration of 0.2 mM for 5 min prior to methylation.

DNAase I footprinting. The same buffer, polymerase concentrations and incubation times were used for footprinting as in the above methylation reactions. Following incubation at 37° with or without ATP and CTP, samples were shifted to 20°C for 1 min, then treated with DNAase (Deoxyribonuclease I, Millipore Corp.) at a final concentration of 5 µg/ml for 30 sec. Reactions were stopped by addition of 30 µl of 3 M ammonium acetate, 0.2 M EDTA, pH 7.5, and 100 µg/ml tRNA followed by 8 volumes of -10°C ethanol. Samples were pelleted, redissolved in 0.3 ml of 0.3 M sodium acetate, ethanol precipitated, rinsed with 80% ethanol, dried under reduced pressure and resuspended in urea-dye mix for gel electrophoresis.

Control experiments with polymerase-DNA complexes. Assays for the fraction of polymerase molecules in the rapid-start configuration (17) showed that approximately 65% of the *B. subtilis* core-sigma and core-sigma + delta molecules which formed binary complexes with SP82 DNA at 37°C were in the open promoter conformation. There was no significant reduction in rapid-start complexes during the shift to 20°C and subsequent incubation with DNAase as described above. The addition of 0.2 mM ATP and CTP had no effect on methylation or DNAase digestion patterns in the absence of polymerase. Identical patterns of methylation and DNAase footprinting patterns were obtained with *B. subtilis* holoenzyme (core-sigma-delta), holoenzyme supplemented with sigma, and preparations of core-sigma supplemented with purified delta peptide.

Core-sigma bound and retained 68% of the Sau3A-Dde 230 fragment in the methylation reactions and core-sigma + delta retained 59% (average of 3 experiments). The maximum extent of dissociation and rebinding during digestion with DNAase I was estimated to be less than 15% (Achberger, unpublished data). In the latter experiments, an excess of single-stranded M13 DNA was added to prevent rebinding of released polymerase to the 230 bp fragment; the dissociation of complexes was followed by means of the filter-binding assay.

Analytical urea gel electrophoresis and autoradiography. Most sequence determinations and all methylation and footprinting experiments were done using a 0.35 mm gel of 8% acrylamide, 0.17% methylenebisacrylamide, 7 M urea with TBE buffer at 1400-1800 volts. The G reaction (14) was used to generate a marker lane for the location of affected bases in the nucleotide sequences. The gels were transferred to a sheet of Whatman 3 mm filter

paper, dried, and frozen at -65°C for autoradiography with Kodak XAR-5 film. To quantitate the intensities of individual bands, films were scanned with a Joyce-Loebel densitometer and the areas of resulting peaks determined using an electronic graphics calculator (Numonics Corp.). The results presented in the next section are averages of 3 separate experiments for each method. Electrophoresis for determination of the ends of the DNA strands and for the RNA was identical to that described above except that the gels were 20% acrylamide-0.66% methylenebisacrylamide and were run at 2000 volts.

RESULTS

Map position of an early SP82 promoter. A DNA fragment of 250 bp in length was identified as containing a SP82 phage early promoter on the basis of the following criteria: 1) it formed binary complexes with unmodified *B. subtilis* RNA polymerase at low enzyme (wt/wt) ratios (8) and 2) this fragment hybridized to a HhaI restriction fragment of SP82 DNA which is transcribed *in vivo* early in infection (8). The fragment, designated Sau3A 250, was mapped by hybridization on the known SP82 genome physical map (Fig. 1) to a HhaI fragment called either Hha f (11) or Hha 32 (4). Hha f contains a single promoter for a major rightward *in vitro* transcript (11). Hybridization of the *in vitro* transcript from Sau3A 250 to the two separated strands of SP82 DNA (data not shown) indicated that transcription is rightward as indicated in Fig. 1.

DNA sequence of the Sau3A-Dde 230 fragment. A fragment of 230 bp was obtained by the cleavage of the Sau3A 250 fragment with DdeI and both strands were sequenced using the chemical method of Maxam and Gilbert (14); the promoter region of this fragment is shown in a later section. The positions of purines near the 5' end of the *in vitro* transcript were determined,

Fig. 1. The position of the sequenced fragment Sau3A 250 on the restriction map of SP82 DNA.

allowing the unambiguous assignment of the transcription initiation site. The promoter sequence includes highly conserved -35 and -10 regions characteristic of *E. coli* promoters (1). This was also found by Lee *et al.* (15) for an early promoter from the related phage SP01. The two *B. subtilis* phage promoters are strikingly similar to one another and to the *E. coli* consensus promoter sequence derived from a comparison of 53 promoters (1).

Probes used to determine interactions. DNAase I is a relatively large probe that introduces single strand nicks in double stranded DNA and has no extreme sequence preferences (6). Protein bound to DNA at a specific site can decrease the access of DNAase to that site resulting in total or partial protection; bound protein can also enhance nicking at certain sites. The mechanisms involved in partial protection and in enhanced nicking are not known, nor is it known whether bases immediately outside the bound protein are vulnerable to DNAase attack.

An example of results obtained in DNAase footprinting experiments with the upper strand is presented in Fig. 2. The characteristic DNAase pattern of cleavage observed in the absence of polymerase (Fig. 2, lane A) was altered by the addition of polymerase (Fig. 2, lanes B-E). However, the presence of delta (Fig. 2, lanes C and E) or ATP and CTP (Fig. 2, lanes D and E) had relatively little effect on the region protected by core-sigma. Differences in the intensities of bands within the protected region were

Fig. 2. Photograph of an autoradiogram from a DNAase I footprinting experiment showing the pattern of protection in the promoter region of Sau3A-Dde 230.

Lane A = control lane in the absence of polymerase; lanes B and C show patterns obtained with core-sigma and core-sigma + delta, respectively in noninitiating conditions; lanes D and E show patterns obtained with core-sigma and core-sigma + delta, respectively, in initiating conditions. Numbers indicate the guanines in the upper strand of the promoter region shown in Fig. 5.

determined by scanning the autoradiograms; the results of these studies and of those for the lower strand are summarized in a later figure (Fig. 5).

To probe polymerase-DNA interactions using methylation, polymerase preparations were incubated with the Sau3A-Dde 230 fragment to allow the formation of complexes and DMS was added; methylated bases (DMS methylates the N7 position of guanine in the major groove and the N3 position of adenine in the minor groove, 18) were located by strand scission and electrophoresis (5). Protection from methylation is presumed to be due to the close proximity of the protein to the methylation site, preventing access by DMS; enhancement probably results from the creation of a hydrophobic pocket by the protein, thereby increasing the local concentration of DMS (5). Both protection and enhancement are interpreted as indicating the close approach of the protein to the affected purine and will be referred to in this paper as "contacts".

Fig. 3 presents data obtained in one experiment on methylation of the upper strand of the sequenced fragment in the presence of polymerase under noninitiating and initiating conditions. The arrows mark the purines for which methylation was either increased or decreased in the presence of polymerase; the effect of methylation was determined by densitometric scanning and quantitation (Table 1). Comparison of the bands marked by arrows in Figs. 3B and 3D demonstrates that delta decreased the number of contacts with purines in the upper strand in noninitiated complexes and had little or no effect on the methylation of purines under conditions of initiation (Figs. 3F and 3G). Similar experiments (data summarized in Table 2 and Fig. 4) indicated that delta had less effect on contacts with purines in the lower strand.

While altered methylation of most affected purines can be observed in a single experiment, quantitation of the intensities of the bands showed some variability ($\pm 15\%$) in the magnitude of the effect in different experiments. Consequently, the intensities of the purine bands within the region of solid protection from DNAase were determined from scans of the autoradiograms of three experiments for each strand. Table 1 (upper strand) and Table 2 (lower strand) present the average values for each purine in these regions expressed as the ratio of the value determined with enzyme (E) to that of the control without polymerase (C). Calculated in this way, $E/C < 1$ indicates that polymerase protected the purine from methylation, $E/C = 1$ indicates no effect, and $E/C > 1$ indicates that polymerase enhanced the susceptibility of the purine to methylation.

Fig. 3. Autoradiograms showing the effects of *B. subtilis* RNA polymerase on the methylation of purines in the upper strand of an early SP82 promoter.

Lanes A, C and E = control lanes without polymerase; lane B = core-sigma, noninitiated complex; lane D = core-sigma + delta, noninitiated complex; lane F = core-sigma, initiated complex; and lane G = core-sigma + delta, initiated complex. The arrows identify affected purines as determined by the quantitation shown in Table 1 (E/C ratios less than 0.8 and greater than 1.25); bases are numbered from the start site of transcription (+1) as shown in Fig. 5.

The results in Table 1 and 2 are diagrammatically represented in Fig. 4. Values falling between the dotted lines, the "cutoff values" (E/C of 1.25 and 0.80), were not considered significant; similar cut-off values were used by Siebenlist and Gilbert in ethylation experiments with *E. coli* polymerase and the A3 promoter of T7 (19). Comparison of the open (upper strand) and closed (lower strand) bars in Figs. 4A and 4B shows that in the noninitiated complexes, the presence of delta decreased not only the number of affected purines but also the extent of enhanced methylation or protection against methylation. Thus, the quantitation of scans (Table 1 and Fig. 4) shows a general decrease in the extent to which the polymerase affected the methylation of purines. Most of the affected purines were clustered around two regions, -40 to -30 and -20 to -10. In contrast, the two

Table 1. Effects of *B. subtilis* RNA polymerase on the methylation of purines in the upper strand of an early SP82 DNA promoter.

Base	Noninitiated		Initiated	
	Core-Sigma	Core-Sigma Plus Delta	Core-Sigma	Core-Sigma Plus Delta
-47	1.00	1.00	1.00	1.00
-45	1.04	0.99	0.87	0.89
-43	0.87	0.99	1.00	0.89
-41	0.93	0.88	0.62*	0.58*
-38	1.00	1.02	1.12	1.07
-37	1.75	1.16	1.82	1.43
-34	2.10	2.07	2.01	1.96
-33	0.53	0.85	0.74	0.70
-27	0.85	0.89	0.53	0.66
-24	0.60	0.64	0.85	0.85
-21	ND	ND	ND	ND
-20	1.84	1.14	2.01	1.76
-19	0.85	0.96	0.94	1.00
-17	0.46	0.69	0.35	0.34
-15	0.48	1.05	0.35	0.36
-13	0.60	0.72	0.66	0.74
-12	0.39	0.88	0.30	0.27
-10	1.11	1.08	1.15	0.94
-9	1.01	1.07	1.04	0.89
-6	ND	ND	ND	ND
-4	ND	ND	ND	ND
-3	1.00	1.00	1.00	1.00
-1	ND	ND	ND	ND
+1	0.85	0.97	0.41	0.34
+3	0.91	0.82	1.14	1.04
+6	0.95	0.88	0.62	0.57

Data are expressed as the ratio of band intensities in autoradiograms in the presence of polymerase (E) relative to controls (C) in the absence of polymerase. The intensities of the bands were determined by scanning the autoradiograms as described in the Methods; values shown are the averages of 3 experiments. Bases are numbered from the start site of transcription (+1). As described in the text, faint adenine bands which were omitted from Fig. 4 are indicated as "ND"; bands omitted from Fig. 5 are marked with an asterisk.

initiated complexes (Figs. 4C and 4D) were very similar and the affected purines were clustered in three regions, -40 to -30, -20 to -10 and -2 to +6.

There are two possible problems with the assignment of protections or enhancements using quantitation: 1) the error involved in quantitating very light bands and 2) the values selected as indicating significantly altered methylation of a purine. Dimethyl sulfate generally methylates guanines more efficiently than adenines and does not methylate all positions equally.

Table 2. Effects of *B. subtilis* RNA polymerase on the methylation of purines in the lower strand of an early SP82 DNA promoter.

Base	Noninitiated		Initiated	
	Core-Sigma	Core-Sigma Plus Delta	Core-Sigma	Core-Sigma Plus Delta
-54	1.00	1.00	1.00	1.00
-53	1.00	1.01	1.00	1.01
-52	0.85	1.00	0.92	1.00
-51	0.82	0.87	1.14	0.99
-50	1.00	0.99	1.12	0.89
-49	0.86	0.91	0.96	1.07
-48	0.91	0.94	0.95	0.96
-46	0.92	0.95	1.12	1.08
-44	1.15	1.16	1.13	1.10
-42	1.09	1.11	1.06	1.05
-40	1.00	1.05	1.19	1.11
-39	0.94	0.94	1.00	1.04
-36	0.96	1.01	1.22	1.54
-35	1.81	1.82	1.75	2.10
-32	0.52	0.68	0.64	0.82
-31	0.70	0.80	0.89	0.91
-30	ND	ND	ND	ND
-29	0.89	0.92	0.96	1.01
-28	0.96	0.97	1.24	1.23
-26	0.94	0.98	0.89	0.92
-25	1.05	1.10	1.05	1.05
-23	0.92	1.00	0.97	0.96
-22	0.99	1.00	1.03	0.99
-18	1.13	1.05	1.53*	1.86*
-16	1.02	1.07	1.23	1.17
-14	2.96	2.83	3.79	2.60
-11	1.69*	1.59*	4.24	2.49
-8	0.80	0.88	0.97	1.12
-7	0.81	0.98	1.09	1.13
-5	0.81	0.94	0.99	0.90
-2	0.86	0.90	3.87*	3.01*
+2	0.91	0.87	0.84	0.80
+4	1.04	1.06	1.20	0.92
+5	0.92	0.90	0.91	0.86
+7	0.90	0.97	0.89	0.82

Data are expressed as described in the legend to Table 1.

Several of the light adenine bands were too close to intense bands to be quantitated accurately (marked "ND" in Tables 1 and 2 and omitted from Figs. 4 and 5). Eight other adenine bands (marked with an asterisk in Tables 1 and 2) were sufficiently well separated to permit quantitation but were so faint that small changes in intensity could lead to large changes in the E/C ratios. Since the effects indicated in Tables 1 and 2 were observed consistently, the data from these faint bands were included in Fig. 4 to show the

Fig. 4. Bar graphs summarizing the effects of *B. subtilis* RNA polymerase on the methylation of purines of the upper strand (open bars) and lower strand (solid bars) of an early SP82 DNA promoter.

A = core-sigma, noninitiated complexes; B = core-sigma + delta, noninitiated complexes; C = core-sigma, initiated complexes; D = core-sigma + delta, initiated complexes. E/C values taken from Tables 1 and 2; bases are numbered from the start site of transcription (+1) as shown in Fig. 5.

maximum number of contacts. A more conservative interpretation of the data is shown in Fig. 5 which omits the data from the faint bands; the major effect of this omission is to decrease the total number of contacts, primarily in the initiated complexes where delta has little or no effect. With regard to the second problem, examination of Fig. 4 and Tables 1 and 2 shows that changing the cutoff values would add or delete a few bases as "con-

Fig. 5. The protection of an early SP82 gene promoter by polymerase from methylation by DMS and attack by DNAase I.

A) Protection patterns of noninitiated complexes in the absence and presence of delta.

B) Protection patterns of initiated complexes in the absence and presence of delta.

The solid and dotted lines indicate regions protected or partially protected, respectively, from cleavage by DNAase I; arrows indicate sites of enhanced cleavage of DNAase I. Circles and half-circles show purines which are protected or partially protected from methylation by DMS; carets show enhanced reaction with DMS. Initiated transcripts were formed in the presence of ATP and CTP; transcription is rightward from +1. T in the above sequence represents 5-hydroxymethyluracil, the thymine analog in SP82 DNA.

tacted" by polymerase but would not affect the overall conclusions concerning the effects of delta on polymerase-DNA complexes.

The effect of delta on polymerase-DNA interactions. A. Noninitiated complexes. We have reported elsewhere (4) that *B. subtilis* holoenzyme (core-sigma-delta) forms highly specific complexes with early SP82 promoters whereas preparations of core-sigma form relatively stable complexes with both promoters and nonpromoter sites. Core-delta does not form filter-retainable complexes with either promoter or nonpromoter sites. If delta is added to core-sigma, the stability of all complexes is reduced, eliminating the formation of stable nonpromoter complexes and restoring the specificity observed with the holoenzyme. The effect of delta on the patterns of pro-

tection from DMS and DNAase attack observed for the core-sigma bound to a promoter are summarized in Fig. 5.

As shown in Fig. 5A, core-sigma provided interrupted protection of the promoter region from attack by DNAase: this region extended from -51 to +17 on the upper strand and -46 to +13 on the lower strand. Under noninitiated conditions, protection from methylation was observed in 8 purines and enhanced methylation at another 5 purines; most purine contacts were clustered in the regions from -40 to -30 and from -20 to -10 and there were more contacts on the upper strand than on the lower strand. General agreement was found between the regions of solid DNAase protection and the sites of close approaches to purines.

When the Sau3A-Dde 230 fragment was incubated with *B. subtilis* core-sigma supplemented with delta, there were slight but consistent differences in the DNAase protection although the limits of protection were the same as for core-sigma. Several significant changes were noted in the methylation pattern upon addition of delta: there were 4 fewer protections, all of the remaining protections were partial and there were 2 fewer purines with enhanced methylation. Both the -40 to -30 and the -20 to -10 regions were affected by the presence of delta. It was interesting that addition of delta resulted in the disappearance of the full protections at -12 and -15 while the partial protection at a nearby position (-13) as well as at -24 and the enhancement at -34 were apparently less markedly affected. However, examination of additional promoters will be required to determine if delta has a differential effect on the interaction of polymerase with specific purines in the promoter. No evidence of interaction with the promoter DNA was seen when delta alone was used in a DNAase footprint or methylation experiment (data not shown). Core-sigma preparations both in the presence and in the absence of delta completely protected the RsaI site at -45 (Fig. 1) from digestion by this restriction enzyme. Additional experiments (e.g., the introduction of alterations in adjacent sequences) will be required to determine the significance of this region to promoter binding.

B. Initiated complexes. ATP and CTP were added during complex formation to permit the synthesis of a short transcript. Highly reproducible results were obtained indicating that the majority of polymerase molecules did not transcribe beyond +3. The extent of the region protected from DNAase was the same as for the noninitiated complexes except that protection on the upper strand extended 3 bases downstream to +20 and 2 bases upstream to -49 on the lower strand (Fig. 5B). This observation, plus the finding

that most close approaches to purines were conserved from the noninitiated state, argues against "sliding" of polymerase upon initiation. Possibly, initiation and synthesis under these experimental conditions is similar to abortive initiation reported for *E. coli* RNA polymerase (20).

Compared to the noninitiated complexes, initiated complexes displayed a dramatic increase in the number of interruptions in protection from DNAase, in the number of sites of enhanced cleavage by DNAase and in the number of purine contacts. As shown in Fig. 5, in the presence of ATP and CTP, 11 of the 13 contacts found for core-sigma in the noninitiated complexes were maintained upon initiation and there were 6 changes in methylation including the formation of 2 new purine contacts around the initiation site (i.e., -2 and +1). Comparison of complexes formed by core-sigma + delta under non-initiating and initiating conditions shows a total of 8 more contacts in the initiated complexes. Only 2 differences were found in the patterns of methylation for initiated complexes plus and minus delta. If all possible contacts are considered (Fig. 4) core-sigma + delta formed 10 (rather than 8) more contacts in the initiated complexes as compared to noninitiated complexes.

DISCUSSION

This investigation revealed that delta affected the patterns of close approaches of *B. subtilis* RNA polymerase to purines (i.e., contacts) in non-initiated complexes formed with an early SP82 promoter. Specifically, more contacts were detected in complexes formed by core-sigma than in complexes formed by core-sigma supplemented with delta and the extent of methylation enhancement or protection of most affected purines was decreased by delta. Based on the results obtained with methylation, we infer that the increase in purine contacts observed with core-sigma indicates a stronger overall interaction with the promoter, since the reaction mixtures were equivalent with respect to the fraction of complexes in the open promoter conformation. The strength of the core-sigma-DNA complexes was confirmed in part by the finding that the loss of delta increased the stability of the complex formed with the promoter on the Sau3A fragment by at least 1.5 fold (Achberger, manuscript in preparation). If it is assumed that the contacts determined between the holoenzyme (core-sigma-delta) and the early promoter used in this study are representative of the interaction required for proper promoter recognition and binding, then the interaction between core-sigma and the promoter is stronger than required or even desirable.

Our earlier report (4) documented the finding that at moderate enzyme-DNA ratios, core-sigma forms stable nonpromoter closed and open complexes; transcription can be initiated from many of these open complexes in the presence of nucleoside triphosphates. In contrast, under the same conditions, the holoenzyme makes stable complexes only at promoter sites, apparently forming nonspecific complexes only transiently, thus allowing proper promoter selection. The earlier work and the results of the present studies support the idea that there are a limited number of interactions possible between holoenzyme and any given binding site on the DNA. If a specific base sequence required for these interactions is not present, stable binding can not be achieved and the complex will quickly dissociate; if a correct promoter sequence is present, polymerase will interact with specific regions of the DNA to stabilize the complex and allow formation of open complexes following the separation of the strands of the DNA. The ability of core-sigma to form significantly more or stronger interactions at nonpromoter sites as well as at promoters would explain the existence of nonpromoter closed and open complexes. This explanation can be tested by isolating fragments of DNA containing nonpromoter sites (sites which can be complexed by core-sigma and from which transcription can occur only in the absence of delta) and examining the DNA sequence and the patterns of polymerase protection from DNAase and DMS. Such studies would also help define the interactions required for open complex formation.

Since our early experiments indicated that delta exerts its effects at the level of DNA binding prior to initiation of transcription, it was anticipated that there would be fewer differences between the initiated core-sigma and holoenzyme complexes than in the noninitiated complexes. If the minimal number of contacts is considered (Fig. 5), 93% (14/15) of the contacts formed by initiated core-sigma + delta were also formed by initiated core-sigma. All but 4 of the 15 contacts formed by core-sigma in initiated complexes were also found for core-sigma in noninitiated complexes. Again, if it is assumed that the contacts observed for the holoenzyme in the two complexes are representative of the correct interactions for each of these stages of transcription, then it is possible that core-sigma forms some interactions with the DNA in the noninitiated complex that are normally found only in the initiated complex. Thus, delta may maintain the polymerase in a conformation that excludes the premature formation of these contacts allowing polymerase to dissociate from nonpromoter or weak promoter sites.

Initiation upon addition of ATP and CTP resulted in increased access to the DNA by DNAase and significantly increased purine contacts by core-sigma + delta. Since the extents of the polymerase-protected region remained relatively unchanged, changes within the protected region suggest an alteration in the conformation of DNA and/or the polymerase. The 2 additional contacts observed at the initiation site in the presence of ATP and CTP are consistent with a closer interaction between this region of the promoter and the polymerase than in noninitiated complexes. It would be of interest to determine if the contacts found in the presence of ATP and CTP exist when a somewhat longer oligonucleotide is synthesized, for example, the 8 base transcript which would be synthesized in the presence of ATP, CTP and UTP.

Comparing the present results with those reported for E. coli RNA polymerase bound to the T7 A3 and the lac UV5 promoters, two points may be noted. First, the DNAase protection patterns of the noninitiated complexes resemble the pattern for E. coli polymerase bound to the lac UV5 promoter (21) and, second, the purine contacts formed by core-sigma most closely resembled those formed by E. coli polymerase with the lac UV5 and T7 A3 promoters (1). Similarities had been reported previously between B. subtilis core-sigma and E. coli holoenzyme. Under initiating conditions, the two enzymes formed nonpromoter complexes at enzyme-DNA ratios and NaCl concentrations at which the B. subtilis holoenzyme (core-sigma-delta) bound specifically to promoters (4,8). Likewise, the B. subtilis core-sigma and E. coli polymerase efficiently transcribed T7 and pBR322 DNAs while transcription of these templates by the B. subtilis holoenzyme was less than 10% that observed with the other two enzymes (4 and unpublished observations).

We had previously described the overall function of delta as the restriction of polymerase binding to promoters specified by the binding-initiation factor (the sigma subunit for the holoenzyme and the 28K peptide for one of the SP82-modified forms of polymerase, 4). Measurements of the rates of dissociation of complexes indicated that delta reduces the stability of all polymerase-DNA complexes and eliminates stable nonpromoter binding (22; Achberger, manuscript in preparation). Our present evidence suggests that this effect is achieved by limiting the interactions between polymerase and DNA, possibly by altering the conformation of the polymerase. In E. coli, promoter recognition and the dissociation of nonspecific complexes are ascribed to a single peptide, sigma. Perhaps the ability of this polymerase to interact with a group of promoters with fairly diverse base sequences is due to a weak "delta function" permitting a greater number of

possible interactions between polymerase and DNA. In contrast, the B. subtilis holoenzyme contains a separate peptide, delta, that appears to limit the polymerase-DNA interactions, thus reducing the number of sites on the DNA that function as promoters. B. subtilis RNA polymerase may achieve transcriptional diversity by employing multiple binding-initiation subunits, each with its own range of specificity.

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