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# Effect of the $\delta$ Subunit of *Bacillus subtilis* RNA Polymerase on Initiation of RNA Synthesis at Two Bacteriophage $\phi$29 Promoters†

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ABSTRACT: Initiation of RNA synthesis by *Bacillus subtilis* RNA polymerase ($\sigma$-43) has been examined at two early promoters of phage $\phi$29: the A2 promoter, which is a weak promoter, and the G2 promoter, which is a strong promoter. The $\delta$ subunit of the polymerase inhibits the rate of initiation at A2, but not G2. In addition, formation of stable complexes by the polymerase at A2, but not at G2, requires the presence of the first two nucleotides of the A2 transcript.

The predominant form of RNA polymerase isolated from vegetative *Bacillus subtilis* cells is associated with a 43 000-dalton $\sigma$ subunit, formerly designated $\sigma$-55 (Gitt et al., 1985), and a 21 000-dalton $\delta$ subunit (Doi, 1982). Although the $\sigma$ factor confers initiation activity upon the enzyme, the $\delta$ subunit, which has no known counterpart in *Escherichia coli*, also influences transcriptional specificity. Pero and her colleagues observed that specific in vitro transcription of phage SP01 middle genes required the host $\delta$ protein (Pero et al., 1975) and similar data have been reported for transcription from SP82 DNA by phage-modified and host cell RNA polymerase (Spiegelman et al., 1978; Achberger & Whiteley, 1981). It has also been demonstrated that $\delta$ limits $\sigma$-43 RNA polymerase/DNA complex formation to restriction fragments of phage $\phi$29 and SP82 DNA that carry promoters recognized by the $\sigma$-43 enzyme (Dickel et al., 1980; Achberger &

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Whiteley, 1981). These data suggested that the *Bacillus subtilis* RNA polymerase requires both σ and δ for efficient promoter recognition and transcription initiation.

Achberger et al. (1982) used DNase I footprinting and methylation protection analysis to investigate the effect of δ upon specific polymerase interactions at an early gene promoter of phage SP82. These studied revealed that δ altered the promoter–polymerase interactions in noninitiated complexes but not in initiated complexes, suggesting that δ acted prior to initiation. The authors therefore proposed that δ altered the conformation of the RNA polymerase to prevent premature formation of initiation-specific contacts with nonpromoter or weak promoter sites.

We have used a run-off transcription assay to investigate the effect of δ on initiation at two φ29 early gene promoters and have found the effect of δ to be promoter dependent. We report here the differences between the two promoters with respect to their strengths and to their response to δ and suggest a role for δ in initiation.

## Materials and Methods

*Isolation of Plasmid DNA.* The plasmid p328-5 contains the A2 promoter from *Bacillus* phage φ29 and has been described previously (Dobinson & Spiegelman, 1985). Plasmid p679 contains a *Hin*dIII to *Hin*fI restriction endonuclease fragment from the right terminus of φ29, cloned into the *Sma*I site of pEMBL8− (Dente et al., 1983). The φ29 fragment contains the G2 promoter (Garvey et al., 1985). The presence of the fragment was confirmed by DNA sequencing. Plasmid-bearing strains were grown as described by Dunn et al. (1979). Cleared lysates were prepared from cells containing p328-5 by the procedure of Clewell and Helinski (1972) with several modifications as described by Dobinson and Spiegelman (1985). Cells containing the plasmid p679 were prepared by the alkaline lysis method described by Maniatis et al. (1982). Plasmid DNA was purified from the lysates by centrifugation in CsCl/ethidium bromide.

*Media.* Plasmid-bearing *Escherichia coli* strains were grown in Luria broth (Maniatis et al., 1982) or in M9 media (Champe & Benzer, 1962) modified as previously described (Dobinson & Spiegelman, 1985).

*Enzymes.* Restriction enzymes were obtained from New England Biolabs or Pharmacia and were used according to the supplier's recommendations.

*RNA Polymerase Purification.* (*A*) *Purification of Holoenzyme* (*Core*/σ/δ). RNA polymerase (σ-43) was purified from *Bacillus subtilis* 168 (Iowa Grain Processing Corp.) as previously described by Spiegelman et al. (1978) by (1) poly(ethylene glycol) (PEG)/dextran phase partitioning, (2) chromatography on Bio-Gel A-1.5M, (3) chromatography over DNA–cellulose, and (4) sedimentation through a glycerol gradient. The procedure was modified as previously described, and the fastest sedimenting fractions from the glycerol gradient activity band were pooled and chromatographed over heparin–agarose (Dobinson & Spiegelman, 1985).

Enzyme activity was assayed by the method of Spiegelman and Whitely (1974), with several modifications (Dobinson & Spiegelman, 1985). Enzyme concentrations stated in the text are in terms of protein concentration [determined by a Lowry assay, as modified by Sandermann and Strominger (1972)] using bovine serum albumin (BSA) (fraction V, Sigma Chemical Co.) as the standard.

(*B*) *Isolation of Core*/σ. Holoenzyme (core/σ/δ) was depleted of δ by DNA–cellulose chromatography of glycerol gradient purified enzyme (Achberger & Whiteley, 1981) or by DEAE-Sephadex chromatography (Spiegelman et al., 1978) of heparin–agarose-purified enzyme.

The amount of δ present in the core/σ and holoenzyme preparations was estimated by electrophoresing the enzyme on sodium dodecyl sulfate (SDS)–polyacrylamide slab gels, as described by Leduc et al. (1982) except that the separation gels contained a 14–20% exponential gradient of acrylamide (McGuire et al., 1974). The gels were stained for 48 h with Coomassie Brilliant Blue R (Sigma Chemical Co.) in a solution of 10% acetic acid/25% ethanol. The gels were then destained with 7.5% acetic acid/25% ethanol, rinsed briefly with 7.5% acetic acid, and scanned with a Quick Scan densitometer (Helena Instruments). The areas under the peaks which corresponded to the δ and α subunits were determined by weight. The amount of α subunit was used as a measure of the amount of core enzyme in the preparation. The δ:α and σ:α ratios were calculated by using an estimated molecular weight of 45 000 for the α subunit, 43 000 for σ (Gitt et al., 1985), and 21 000 for the δ subunit (Doi, 1982). (The σ:α ratio was used to estimate the amount of active enzyme in preparations.) The amount of δ in the holoenzyme preparations ranged from 57% to 66% and was a maximum of 30% in enzyme which had been depleted of δ. Core/σ was reconstituted with δ by incubating δ with core/σ for 2 min at 37 °C prior to adding the enzyme to the transcription reactions.

*Purification of the δ Subunit.* δ was purified from pooled δ-containing fractions of glycerol gradients (from step 4 of the holoenzyme purification). The combined fractions were heated at 65 °C until the solution became flocculent (30–60 min) and centrifuged at low speed to remove the resulting precipitate. The supernatant contained δ which is heat stable (M. Hilton, personal communication), that could be further purified by chromatography over DEAE-Sephadex (Spiegelman et al., 1978). It should be noted the 65 °C heat treatment had no effect on the activity of δ when compared to δ isolated without heat treatment.

The amount of δ in the preparations was estimated by running samples on SDS–polyacrylamide gels, with known amounts of BSA as standards. The gels were stained with Coomassie Blue and scanned, and the areas under the peaks which corresponded to BSA and δ were quantitated as described above. The area of the BSA peaks was used to generate a standard curve, and the amount of δ was estimated by comparison of the δ peaks with the standard curve. The δ preparations were free from protease and nuclease activity (data not shown).

*In Vitro Transcription Assays.* Run-off transcriptions were done in 40- or 100-μL volumes essentially as previously described (Dobinson & Spiegelman, 1985), except tha⁺ the buffer contained 40 mM tris(hydroxymethyl)aminomethane hydrochloride) (Tris-HCl), pH 7.9 20 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 48.5 mM NaCl, and 3% glycerol, and the volumes of the elongation reactions were 40 or 100 μL. The transcription products were electrophoresed through denaturing polyacrylamide gels (Dobinson & Spiegelman, 1985), and the incorporation of radioactivity into specific transcripts was quantitated by Cerenkov counting of gel slices containing the transcripts.

## Results

We investigated the effect of the δ subunit of the *B. subtilis* RNA polymerase on transcription from the φ29 A2 and G2 promoters using an in vitro transcription assay similar to that described previously by Stefano and Gralla (1980, 1982). Briefly, the assay was carried out by first incubating the RNA polymerase with a promoter-containing DNA template that had been cleaved at restriction site downstream from the

Table I: DNA Sequences at the A2 and G2 Promoters[a]

| | |
|---|---|
| A2 | ATTTGGTTAATCAACGTTTACAAAGTGAACAGAAAGTGTTAAACTATATAGAGACACA |
| G2 | AATTTGTCGAAAAGGGTAGACAAACTATCGTTTAACATGTTATACTATAATAGAAGTAA |
| Consensus | TTGACA - 16 bp ± 1 - TATAAT |
| Position | 123456 123456 |

[a] DNA sequences [from Garvey et al. (1985)] are compared with the consensus promoter sequences (Rosenberg & Court, 1979; Moran et al., 1982; Hawley & McClure, 1983) for promoters recognized by *B. subtilis* $\sigma$-43 and *E. coli* $\sigma$-70 RNA polymerases. The −10 and −35 sequences are underlined. Bases in the consensus sequence which are underlined appear to be highly conserved in *B. subtilis* (Moran et al., 1982). Bases marked with an asterisk are changed from the consensus, and the initiation point is marked with a solid dot.

promoter. Heparin and ribonucleotides were then added to the reaction, and enzyme which had formed a heparin-resistant complex with the promoter could synthesize a run-off transcript. The assay thus measured the formation of heparin-resistant complexes by following synthesis of the run-off transcripts. The nucleotide sequences of the two promoters used in this study are shown in Table I. The −10 and −35 consensus sequences are underlined, and the initiation positions are marked with a solid dot. The nucleotide sequence at the A2 promoter (Dobinson & Spiegelman, 1985) indicated that the transcript should start with pppAGAC. At the G2 promoter, the 5′ end of the transcript should be pppGAAGT (Garvey et al., 1985).

During the course of our work, we observed that the $\sigma$-43 RNA polymerase was inefficient at forming heparin-resistant complexes when incubated alone with the A2 promoter (see Figure 1, for example). The kinetics and extent of heparin-resistant complex formation in the absence of nucleotides were therefore compared to complex formation in the presence of (1) ATP or (2) ATP and GTP. Complex formation was measured by the run-off transcription assay, in which the amount of transcription was quantitated as a function of the time between addition of polymerase to the reaction and the addition of heparin and remaining nucleotides.

Figure 1a shows the autoradiogram of a gel of RNA products synthesized by the RNA polymerase in the presence of combinations of the nucleotides. The complex formation reactions were carried out for the times indicated in the figure legend. The amount of radioactivity incorporated into the A2 transcripts was also plotted against the amount of time allowed for complex formation (see Figure 1b). When complex formation was carried out in the absence of nucleotides (Figure 1, lanes A1–A5), the A2 transcript was synthesized at very low levels, implying that under those conditions the RNA polymerase remained in a state which was sensitive to inactivation by heparin. Incubating the enzyme with ATP and the template for up to 10 min (Figure 1a, lanes B1–B5) yielded essentially the same level of transcripts as when the enzyme and template were incubated together, and still less than 0.05 transcripts per promoter. The addition of ATP and GTP to the initiation reaction resulted in a 10-fold increase in the maximum yield of heparin-resistant complexes (Figures 1a,b), suggesting that complexes were stable at A2 only when they contained at least a dinucleotide. The other implication of these results is that complexes formed with or without ATP were converted to heparin-resistant complexes at a very slow rate when heparin and the remaining nucleotides were added.

*Formation of Heparin-Resistant Complexes at the G2 Promoter.* The plasmid carrying the $\phi$29 G2 promoter (Sogo et al., 1979; Davison et al., 1979, 1980; Garvey et al., 1985)

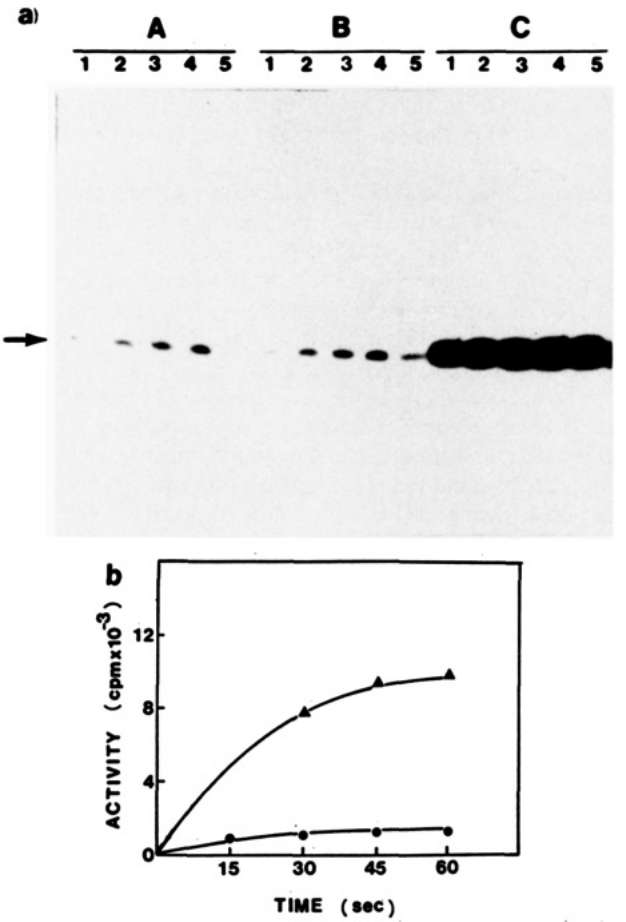


FIGURE 1: Nucleotide requirements for heparin-resistant complex formation at the A2 promoter. (a) Autoradiogram of a polyacrylamide gel of the transcription products from the assay for complex formation. (b) Plot of data from initiation time course (15–60-s time points): (●) complex formation in the absence of nucleotides or presence of ATP; (▲) complex formation with ATP and GTP. The ordinate represents the amount of [$\alpha$-$^{32}$P]UTP incorporated into the A2 transcripts. RNA polymerase (9 nM; lacking the $\delta$ subunit) was incubated with DNA (1.7 nM) and the following ribonucleoside triphosphates: (A) no nucleotides; (B) ATP; (C) ATP and GTP, Initiation was allowed for 15 s (lane 1), 30 s (lane 2), 45 s (lane 3), 60 s (lane 4), or 10 min (lane 5). Heparin and the ribonucleotides which were not present during complex formation were added to allow synthesis of transcripts. Transcripts (237 bases long) were separated on denaturing gels as described under Materials and Methods. Concentrations of nucleotides were 400 $\mu$M for ATP and GTP during the binding reaction. During the elongation phase, the CTP concentration was 400 $\mu$M; UTP, which also included [$\alpha$-$^{32}$P]UTP at 1–5 $\mu$Ci/reaction, was present at a concentration of 10 $\mu$M, and the concentrations of ATP and GTP were 380 $\mu$M (Dobinson & Spiegelman, 1985). The nucleotide sequence at the A2 promoter initiation site indicates that the A2 transcript would begin with pppAGAC.

can be cleaved downstream of the G2 promoter using the restriction enzyme *Hin*dIII. Transcription of the linearized plasmid with the $\sigma$-43 RNA polymerase yields a run-off RNA of approximately 120 bases, which can be easily distinguished from the A2 transcript on polyacrylamide gels. The sequence of the G2 promoter indicates that the transcript should begin pppGAAGT (see Table I). The formation of heparin-resistant complexes at the G2 promoter in the absence of nucleotides was compared to complex formation in the presence of (1) GTP, the initiating rNTP (Davison et al., 1980; Garvey et al., 1985), or (2) ATP and GTP. When holoenzyme was incubated with the G2 template in the absence of nucleotides, the rate of complex formation and the yield of complexes were essentially the same as when GTP or both ATP and GTP were included in the binding step (Figure 2). Unlike A2, the

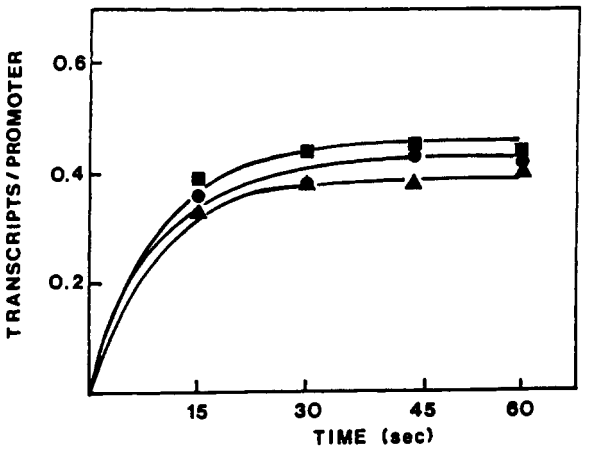


FIGURE 2: Nucleotide requirements for stable complex formation at the G2 promoter. The RNA polymerase (core/$\sigma$/$\delta$) was incubated with *Hin*dIII-treated G2 promoter template (1.7 nM) in the absence of nucleotides (●), in the presence of GTP (▲), or with ATP and GTP (■) for the times indicated. Heparin and the remaining nucleotides were then added to allow the synthesis of transcripts from complexes which had formed during the complex formation reaction. Complex formation is expressed as the number of transcripts synthesized per G2 promoter. The enzyme concentration was 11 nM. Nucleotide concentrations are indicated in the legend to Figure 1, and the nucleotide sequence at the G2 promoter predicts a transcript beginning with pppGAAGT.

formation of heparin-resistant complexes at G2 is rapid enough that production of run-off transcripts under challenge by heparin does not require prior initiation.

*Stability of Complexes at the A2 Promoter.* The sensitivity of complexes of polymerase and DNA at the A2 promoter to heparin suggested that such complexes might be unstable. Competition assays were carried out to measure the stability of these complexes. The assay was performed as follows: the RNA polymerase was first incubated with p328-5 DNA. After 5 min, plasmid carrying the $\phi$29 G2 promoter was added, and at 15-s intervals after the addition of the G2 promoter, DNA samples were removed and added to ATP and GTP to allow enzyme bound to either of the promoters to form initiation complexes. The incubation with ATP and GTP was carried out for 10 min before heparin, CTP, and UTP were added to allow elongation from the complexes.

The results of one competition experiment are shown in Table II. When the G2 and A2 templates were incubated together in the presence of the RNA polymerase, the level of transcription from A2 was 0.02 transcript per enzyme while 0.29 transcript per enzyme was synthesized from G2. The G2 template thus competed very effectively with the A2 promoter for the available enzyme when the two templates were added simultaneously. The G2 promoter also acted as an efficient competitor when it was added to preformed A2/RNA polymerase complexes. Within 15 s after the G2 template was added to the A2 complexes, the distribution of complexes at the two promoters was the same as when the two templates were simultaneously incubated with the enzyme. The enzyme bound at the A2 promoters dissociated so rapidly that it was not possible to ascertain a dissociation rate.

The data from the competition assay yielded two additional pieces of information about transcription from the A2 and G2 promoters. First, when the RNA polymerase was not incubated with the A2 template prior to initiation, approximately 0.2 A2 transcript was synthesized per enzyme (Table II, condition 1). The enzyme activity decreased by 60%, to 0.08 transcript per enzyme, when the A2 template and enzyme were preincubated together for 5 min (Table II, condition 2), suggesting that the enzyme was inactivated when heparin-

Table II: Template Competition Assay[a]

| assay conditions | template | prebinding | transcripts/enzyme A2 | transcripts/enzyme G2 |
|---|---|---|---|---|
| 1 | A2 | − | 0.21 | |
| | A2/G2 | − | 0.05 | 0.38 |
| 2 | A2 | + | 0.08 | |
| | A2/G2 | + | 0.02 | 0.29 |
| 3 | A2/G2 | + | 0.02 | 0.21 |

[a] The effect of a competitor (G2) template on transcription from the A2 template in a single-round transcription assay was measured under the following conditions: (1) Standard productive transcription conditions were used (10-min AG initiation followed by a 10-min elongation reaction) with A2 alone or with A2 and G2. (2) The initiation reaction was preceded by a 5′15″ binding reaction during which time A2 or A2 and G2 were incubated with the RNA polymerase. (3) A2 was incubated with the RNA polymerase for 5 min, then G2 was added, and the binding reaction was continued for an additional 15 s before initiation was allowed to occur. The concentration of each DNA template was 8.5 nM, and the active enzyme concentration (determined as described under Materials and Methods) was 5 nM.

resistant initiation complexes could not be formed. We assume that when both A2 and G2 complexes are incubated together, the formation of complexes at G2 would partially protect enzyme which dissociated from A2. However, as can be seen by comparing the level of transcripts per enzyme from both A2 and G2 under condition 1 with the levels seen under conditions 2 and 3, there was some loss of transcription activity during prebinding in the presence of A2 and G2. Second, more transcripts were synthesized from the G2 promoter than from the A2 promoter, supporting a previous report by Davison et al. (1980) than in vitro transcription from the G2 promoter was much more efficient than transcription from the A2 promoter at low enzyme concentrations. The two promoters are therefore quite different with respect to their strengths and to the nucleotides required for formation of heparin-resistant complexes.

*Effect of δ on Initiation at the A2 and G2 Promoters.* The run-off transcription assay was used to investigate the effect of $\delta$ on complex formation at the two $\phi$29 promoters. In these assays, transcription by RNA polymerase that had been depleted of $\delta$ (core/$\sigma$), as described under Materials and Methods, was compared to transcription by core/$\sigma$ which had been reconstituted with purified $\delta$, to ensure that any observed differences in the initiation kinetics were not due to differences between enzyme preparations.

In the experiment shown in Figure 3, complex formation at the A2 promoter was measured as a function of the time allowed for initiation. In this particular experiment, approximately 0.8 transcript per promoter was synthesized by core/$\sigma$ following a 60-s initiation. When core/$\sigma$ was reconstituted with $\delta$, the number of complexes formed during a 1-min initiation dropped by 50% to approximately 0.4 transcript per promoter. The inhibitory effect of $\delta$ was very specific with respect to the time at which $\delta$ was added to the transcription. When added subsequent to complex formation, $\delta$ had no effect on the level of transcription (see Figure 3). To inhibit transcription from A2, $\delta$ must therefore be present during the formation of enzyme/DNA complexes.

Kinetic studies of promoter-specific interactions by the *E. coli* RNA polymerase have shown that the formation of open complexes at some promoters exhibits pseudo-first-order kinetics under conditions of enzyme excess (Stefano & Gralla, 1980; Hawley & McClure, 1980; McClure, 1980). The data presented in Figure 4 show that the formation of initiation complexes at A2 also followed pseudo-first-order kinetics and that the addition of $\delta$ to core/$\sigma$ inhibited the rate at which

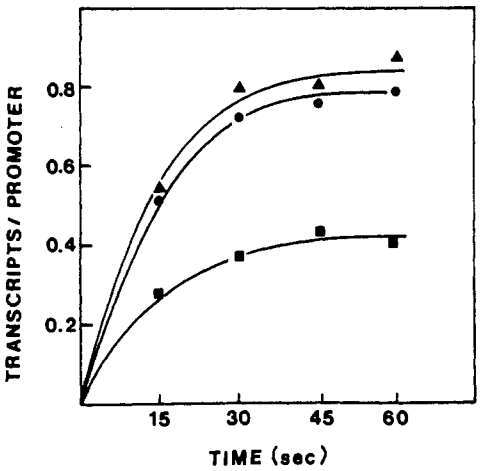


FIGURE 3: Effect of $\delta$ on complex formation at A2. RNA polymerase was incubated with the A2 promoter template, ATP, and GTP for the indicated time intervals. Aliquots of the reaction were then added to heparin, CTP, and UTP to allow elongation of transcripts from complexes formed during the initiation reaction. (●) Core/$\sigma$; (■) core/($\sigma$ + $\delta$); (▲) core/$\sigma$ present during formation of initiation complexes, and $\delta$ added to the reaction with the heparin, CTP, and UTP. The template DNA concentration was 1.7 nM, and the enzyme concentration was 30 nM. $\delta$ was added to core/$\sigma$ at a ratio of 0.8 mol of $\delta$/1 mol of core/$\sigma$.

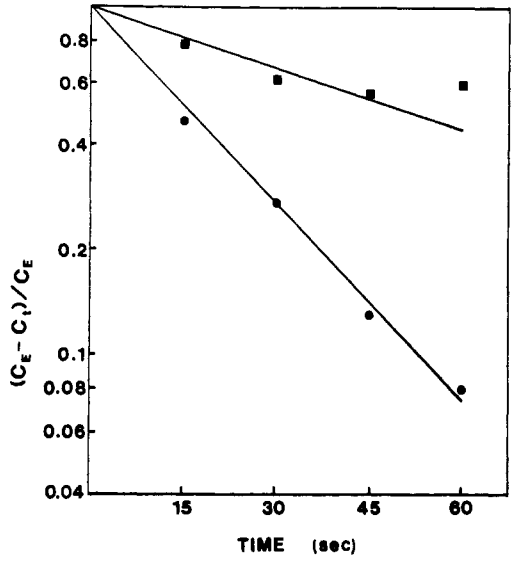


FIGURE 4: Semilogarithmic plot of the data from the AG initiation time courses shown in Figure 3. The ordinate represents the fraction of unoccupied promoter sites remaining after the period of initiation indicated, calculated from the level of transcription ($C_t$) obtained after initiation time $t$ and the maximum level of transcription ($C_E$) obtained after 10 min of initiation. The promoter concentration was 1.7 nM, and the RNA polymerase concentration was 9 nM. (●) Core/$\sigma$; (■) core/($\sigma$ + $\delta$) (3.6 mol of $\delta$/mol of enzyme).

initiation complexes formed at A2.

It has been predicted that $\delta$ would inhibit transcription from weak promoters, as well as from nonspecific sites on the DNA, but have little effect on transcription from strong promoters (Achberger et al., 1982). Transcription from the G2 promoter appears to be much more efficient in vitro than transcription from the A2 promoter (Table II; Davison et al., 1980). It was therefore of interest to determine whether under the conditions of our assay system, $\delta$ would affect complex formation at G2. In order to directly compare the data with those from the A2 promoter, ATP and GTP were included in the initiation step. Since the RNA synthesized from G2 begins with the sequence pppGAAG (Table I), the RNA polymerase should be able to form a ternary complex at the G2 promoter, similar to the

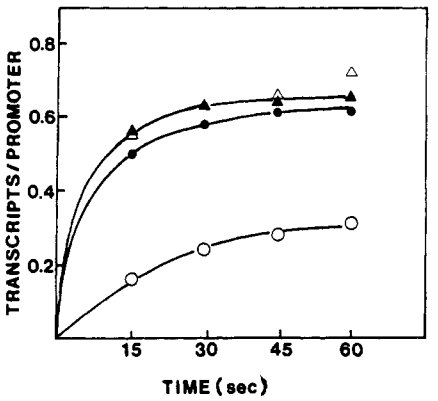


FIGURE 5: Effect of $\delta$ on initiation from the $\phi$29 A2 and G2 promoters. Core/$\sigma$ was incubated with (open symbols) or without (closed symbols) $\delta$ (at a ratio of 0.9 mol of $\delta$/1 mol of enzyme) in the presence of ATP, GTP, and the A2 or G2 promoter for 15–60 s prior to elongation. Formation of AG-initiated complexes is expressed as the number of transcripts synthesized per A2 or G2 promoter. (△) G2 promoter, (○) A2 promoter. Promoter concentrations were 1.7 nM. Enzyme concentration was 49 nM.

ternary complex formed at A2, in the presence of ATP and GTP. The effect of $\delta$ on complex formation at G2 is shown in Figure 5. As a control, the effect of $\delta$ was also measured simultaneously with the A2 promoter. $\delta$ had no effect on complex formation at the G2 promoter, while the final level of complex formation at the A2 promoter was inhibited by approximately 50%.

## DISCUSSION

The initiation complex assays we have used measure the rate at which RNA polymerase proceeds through what are certainly many steps in its association with the promoter before reaching a stage where the transition to elongation occurs faster than inactivation by heparin. At such a stage, the enzyme will yield transcripts when challenged by nucleotides and heparin and thus is what we term heparin resistant. Work with *E. coli* RNA polymerase has shown that the initiation reaction involves an open complex in which the polymerase is bound to the promoter and the DNA helix is unwound [reviewed in Chamberlin (1976), von Hippel et al. (1982), and McClure (1985)]. For *E. coli*, many open complexes are stable, and the enzyme in those complexes is resistant to heparin inactivation (Miller & Burgess, 1978). Although the heparin-resistant, open complex state itself may be heterogeneous (McClure, 1985), it serves to define a stage in the initiation reaction.

When *B. subtilis* RNA polymerase was incubated with DNA fragments containing the A2 promoter, the complexes which formed were very sensitive to heparin, even at the low concentrations we used (5 $\mu$g/mL). Other *B. subtilis* polymerase/promoter complexes have been shown to be sensitive to as little as 2.5 $\mu$g/mL heparin (Talkington & Pero, 1978). The unusual sensitivity to heparin at the A2 promoter, and possibly other promoters, could be explained if the polymerase does not reach the open complex stage very rapidly, or if the open complex state is not as stable as it is for the paradigmatic *E. coli* promoter/polymerase complexes.

At the A2 promoter, the preferred initiating nucleotide is ATP (Dobinson & Spiegelman, 1985), so we expected that adding ATP to the incubation of polymerase and promoter might favor the formation of complexes capable of initiating and elongating RNA when challenged with heparin. However, formation of heparin-resistant complexes from the A2 promoter required both ATP and GTP. In the presence of ATP

and GTP, the polymerase can synthesize the trimer pppAGA (Dobinson & Spiegelman, 1985), suggesting that heparin-resistant complex formation at A2 requires synthesis of at least one and possibly two phosphodiester bonds. Two lines of evidence suggest that the sensitivity of A2 complexes to heparin results from their instability. First, previous reports have indicated that *E. coli* RNA polymerase looses activity during in vitro reactions, probably due to denaturation, and that DNA can protect the enzyme if stable complexes between the polymerase and promoters on the DNA can be formed (Hinkle & Chamberlin, 1972; Stefano, 1981). We routinely observed that the number of transcripts produced decreased with the incubation time when initiation reactions employing the A2 promoter contained either no nucleotides or only ATP, but not when both ATP and GTP were included. The loss of activity suggested that the complexes formed in the absence of both ATP and GTP were unstable, leaving the enzyme sensitive to denaturation. Second, the data we presented in Table II are a direct measure of complex stability at the A2 promoter and demonstrated that such complexes dissociated very rapidly.

Although the characteristic of requiring synthesis of at least a dinucleotide for promoter/polymerase complex stability such as was seen at A2 is unusual, it is not unique. A similar requirement has been seen for formation of heparin-resistant complexes at the tyrosine tRNA promoter in *E. coli* (Kupper et al., 1976). Furthermore, Le Grice et al. (1986) reported that initiation reactions using *Bacillus* RNA polymerase which were primed with the first two nucleotides yielded more RNA than when primed with a single nucleotide. Finally, we have observed that formation of heparin-resistant complexes at *B. subtilis rrn*B promoters also requires the first two nucleotides (Webb and Spiegelman, unpublished results).

In contrast to those formed at the A2 promoter, the RNA polymerase/promoter complexes at G2 were much more like those formed by *E. coli* RNA polymerase at strong promoters. Such complexes formed very rapidly and were stable (see Table II). Furthermore, the formation of the heparin-resistant complexes at G2 did not require addition of nucleotides. The difference between the initiation reactions at A2 and G2 promoters emphasizes the variability in initiation requirements at different promoters which, as has been pointed out by others, might have regulatory functions (McClure et al., 1978; McClure, 1985; Le Grice et al., 1986).

*Effect of the δ Subunit on Complex Formation at the A2 and G2 Promoters.* There is considerable evidence that δ acts prior to initiation (Spiegelman et al., 1978; Achberger et al., 1982). The data from the single-round transcription assay shown in Figure 3 demonstrated that the formation of heparin-resistant complexes at the A2 promoter was inhibited by δ. There is no trivial explanation for the inhibition since the δ preparation was free of DNase, RNase, and proteolytic activity (data not shown) and there was no inhibition if δ was added to the reaction after initiation complexes had been formed. These data suggest that δ directly affects some step in the initiation pathway. δ does not appear to be a general inhibitor of promoter-specific transcription but rather a promoter-specific modulator, since complex formation at the G2 promoter was not affected by the presence of δ (see Figure 5). This result supports the hypothesis that δ would not inhibit complex formation at strong promoters (Achberger et al., 1982).

It has been proposed that δ plays a role in enhancing the specificity of the *B. subtilis* RNA polymerase by inhibiting the formation of stable complexes at nonspecific sites on the *Bacillus* chromosomal DNA (Achberger et al., 1982; Doi, 1982). The implication of this hypothesis is that polymerase interactions with nicked DNA or nonpromoter sequences interfere in some way with promoter location. It would therefore be expected that enzyme which is associated with δ would transcribe promoters more efficiently, while nonspecific transcription would be reduced. It was observed, however, that core/($\sigma$ + δ) did not form heparin-resistant complexes any more rapidly at the G2 promoter than did core/$\sigma$ (see Figure 5). This result suggests that nonspecific complexes formed with the plasmid by core/$\sigma$ are not kinetically important, at least with respect to the ability of the enzyme to locate available promoter sites.

*Role of δ in Complex Formation at the A2 and G2 Promoters.* On the basis of our data and previous information about δ, the role of δ can be considered to include a modulating function to aid the RNA polymerase in discriminating between specific promoters (Williamson & Doi, 1978; Achberger et al., 1982). In the case of the G2 promoter, the formation of heparin-resistant complexes seems to be analogous to complex formation at the *E. coli lac*UV5 promoters where RNA polymerase associates with the promoter and undergoes a series of conformational changes involving one or more closed complex intermediates. The closed complex is then converted to an open complex that does not readily dissociate (McClure, 1985). Previous data (Spiegelman & Whiteley, 1979; Achberger et al., 1982; Hyde et al., 1986) indicate that δ is released from the complex at some point during this sequence of events and in the presence of nucleotides the enzyme is subsequently able to initiate transcription. There is no evidence to suggest that enzyme which is not associated with δ binds to promoters and initiates transcription via a different pathway from the one used by enzyme containing δ.

To account for the observations we have reported, we propose that at the A2 promoter, the core/$\sigma$ closed complex undergoes a direct transition to an unstable open complex that can subsequently form a heparin-resistant initiation complex in the presence of ATP and GTP. Core/$\sigma$/δ may bind to the A2 promoter but does not readily form open complexes which can initiate transcription. We envisage two general models for the effect of δ at the A2 promoter. First, it is possible that δ inhibits the rate of the reaction by altering the conformation of the enzyme such that the enzyme does not readily undergo one or more of the steps in the pathway. Alternatively, the release of δ may be associated with the rate-limiting step in the initiation reaction. To account for the differences between the A2 and G2 promoters, we suggest that δ is efficiently released from complexes at the G2 promoter but not from complexes at the A2 promoter.

*Comparison of the Sequences of the A2 and G2 Promoters.* The data presented here have shown that the $\phi$29 A2 and G2 promoters are quite different with respect to promoter strength, the nucleotide requirements for heparin-resistant complex formation, and the effect of δ on stable complex formation. The sequences of the two promoters were examined to see if differences in the structures of the promoters could account for the differences in the way in which the RNA polymerase interacts with the two promoters. The sequences of the two promoters and the *E. coli*/*B. subtilis* consensus sequence (Rosenberg & Court, 1979; Moran et al., 1982; Hawley & McClure, 1983) are shown in Table I.

There are several outstanding sequence differences between the A2 and G2 promoters. Both the A2 and G2 promoters differ from the consensus at one position in the −35 sequence. The base change in the A2 promoter is at the highly conserved G residue at position 3 in the −35 region, and all *E. coli* of

the promoter mutations at that position are "down" mutations (Hawley & McClure, 1983). Furthermore, the sequences of nine *Bacillus* promoters compiled by Moran et al. (1982) all had a G residue at position 3 in the −35 region. The −35 sequence of the G2 promoter differs from consensus at the T residue in position 2. This base is very highly conserved in both the *E. coli* and *B. subtilis* promoters that have been analyzed (Moran et al., 1982; Hawley & McClure, 1983). It is not clear, however, how a base change at this site would affect initiation since the G2 promoter appears to be a fairly strong promoter and mutations at this site in *E. coli* promoters may either enhance or diminish promoter activity (Hawley & McClure, 1983). The −10 regions of the two promoters are identical except for position 3. This site is weakly conserved in *E. coli*, but eight of the nine *Bacillus* promoters analyzed by Moran et al. (1982) had a T residue at that position. The A2 promoter, on the other hand, has an A residue at that site.

As well as having differences in −10 and −35 regions, the A2 and G2 promoters also differ in other respects, for example, the length of the spacer sequence between the −10 and −35 regions. Stefano and Gralla (1982) have shown that the rate of open complex formation at the *E. coli lac* promoter is affected by the length of this spacer region. The promoters reported by Moran et al. (1982) indicated that a 17 base pair (bp) spacing was most favorable in *Bacillus*, although few detailed kinetic studies have been carried out. On the other hand, the G2 promoter, which has an 18 bp spacing, is clearly a stronger promoter than A2 which has a 17 bp spacing. A *Bacillus* promoter with an 18 bp spacing studied by Le Grice et al. (1986) was also an effective template in vitro. Finally, the 5′ flanking regions of A2 and G2 promoters differ in sequence. Both promoters have high A·T content, reflecting the phage $\phi$29 genome. Flanking sequences have been reported to stimulate transcription for a number of promoters. In one case, an A·T-rich region was removed from a promoter without decreasing the in vitro promoter strength (Le Grice et al., 1986), while in others (Lamond & Travers, 1983; Pethö et al., 1986) the A·T-rich region stimulated transcription. On the whole, we cannot point to any one difference in the DNA sequences of A2 and G2 which causes the variability in initiation characteristics. Indeed, it seems likely that the variation may be the result of interaction with several blocks of sequences.

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# Covalent Attachment of an Alkylamine Prevents the B to Z Transition in Poly(dG-dC)†

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ABSTRACT: Covalent complexes of *n*-butylamine and double-stranded poly(dG-dC) were prepared by coupling the amine to exocyclic amino groups of guanine bases with $CH_2O$. Neither the absorption spectrum above 230 nm nor the $s^0_{20,w}$ of the complexes in low to moderate ionic strength solvents, freed of excess unreacted reagents, differs significantly from that of unreacted poly(dG-dC) or a control which had been exposed only to $CH_2O$. In contrast, the CD spectra are profoundly altered. The minimum at 252 nm becomes more negative, and the rotational strength of the positive band above 260 nm is reduced as a linear function of the extent of amine attachment. At 0.22 mol of amine per mole of nucleotide, the transformation is similar to that observed by others in poly(dG-dC) when complexed to core histones in reconstituted core particles or in concentrated LiCl solvents at temperatures below the B → Z transition. Sedimentation studies reveal that these changes in the circular dichroism (CD) spectra reflect secondary structural effects rather than the formation of aggregates or $\psi$ type structures. Raman spectra reveal, however, that these secondary structural changes must occur within the B family as the amine complex retains B backbone geometry. The conformation produced by the attachment of the amine is probably a higher winding angle (overwound) B variant. If the substitution level is above 0.06 mol of amine per mole of nucleotide at 27 °C, exposure of the complex to 4 M NaCl does not appreciably alter the absorbance spectrum and only causes a further depression of the positive band and an increase in negative rotational strength of the negative band of the CD spectrum, indicating a failure to transform to the Z structure. These spectral properties are unchanged by heating at 60 °C for 1 h. In fact, the solution of derivative in 4 M NaCl must be heated above 80 °C, with consequent total loss of covalently bound amine, before transformation to the Z form occurs. Examination of a series of such derivatives of different amine content between pH 7 and 10.7 in 4 M NaCl reveals that it is the charge on the amine rather than its steric properties which prevents transformation to the Z form. The B → Z transformation will not ensue until the *charged* amine content has been reduced to an *average* of ca. 0.05 mol of positive charge per mole of nucleotide, or ca. 1 in 20 bases. Control samples poly(dG-dC) exposed only to $CH_2O$ in the reaction mixture show the typical B → Z transformation in CD and absorption spectra in going from 20 mM to 4 M NaCl. The stabilization of the B form of poly(dG-dC) by the attachment of a positively charged amine in the minor groove thus appears to be a thermodynamic rather than a kinetic phenomenon. These derivatives should be useful in elucidating the role of elelctrostatic factors in the B → Z transition.

We have previously found that primary amines can be covalently attached to bases in linear (Chen et al., 1981) and covalently closed duplex DNA (Kilkuskie, 1982; Kilkuskie et al., 1982) by coupling with $CH_2O$ at concentrations of the latter insufficient to cause significant denaturation or cross-linking of bases during the time course of the reaction. Although exhaustive dialysis (which removes the excess amine and formaldehyde) will result in some reversal of the reaction, a significant fraction of bases retains amine and formaldehyde in a mole ratio of 1:1 of amine/formaldehyde. Derivatization appears to have the same conformational effect on random sequence DNA as do increases in the electrolyte content of the aqueous solvent or complexion with core histones. This effect has been shown by our laboratory (Chen et al., 1981) to be due to a positive charge on the derivatized base product rather than the steric properties of the formaldehyde/amine adduct. Since the linear charge density and the ion content of the grooves of duplex DNA can be varied by controlling the extent of amine attachment to the final product, these derivatives can be used to elucidate the role of electrostatics in modulating the conformational characteristics of DNA. We have correspondingly undertaken an extensive series of investigations of their properties. The results of our studies with random sequence DNAs form the background of this current study. Because of their relevance, these are summarized below.

A large number of amines will undergo this reaction, although the major share of the characterization work has been

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