A KINETIC ANALYSIS OF TRANSCRIPTION INITIATION BY THE *BACILLUS SUBTILIS* SIGMA-43 RNA POLYMERASE. THE EFFECT OF THE DELTA SUBUNIT

by

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ABSTRACT

The initiation of transcription by the *Bacillus subtilis* sigma-43 RNA polymerase at two *Bacillus* phage ϕ29 promoters and the effect of the delta subunit on initiation have been investigated by an *in vitro* kinetic analysis. The templates for the analysis were plasmids which carried the ϕ29 A2 or G2 promoter. The cloning and localization of the A2 promoter are reported here.

The kinetics of RNA synthesis initiation were examined using a single-round run-off transcription assay in which multiple initiation events at a single promoter were inhibited with heparin. It was observed that the formation of heparin-resistant complexes at the A2 promoter required the presence of the initiating nucleotides, while the RNA polymerase alone was able to form heparin-resistant, non-initiated complexes at the G2 promoter. The G2 promoter was also shown by a competition assay to be a stronger promoter than A2.

The effect of the delta subunit on complex formation at the two promoters was investigated with the single-round transcription assay. Delta had no effect on the formation of initiation complexes at the G2 promoter but lowered the rate and extent of complex formation at the A2 promoter.

The effect of delta on the kinetic parameters of complex formation at the A2 promoter was also investigated. The data suggested that delta affects the efficiency with which the enzyme/promoter complexes undergo the

transition(s) to a complex from which RNA synthesis can be initiated, although other interpretations were possible. A model for the effect of delta is proposed, in which it is postulated that the release of delta from the enzyme/promoter complex is essential for initiation. Enzyme which is associated with delta can interact with both the A2 and G2 promoters but complexes at the weaker A2 promoter do not efficiently release delta, thus slowing the formation of initiation complexes.

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LIST OF ABBREVIATIONS

b.p.	Base pair
BSA	Bovine serum albumin
cpm	Counts per minute
EDTA	Ethylenediaminetetra-acetic acid
kb	Kilobasepairs
kd	Kilodalton
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
rNTP	Ribonucleoside triphosphate
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TCA	Trichloroacetic acid

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INTRODUCTION

In Escherichia coli and other prokaryotes transcription is mediated by a DNA-dependent RNA polymerase which is responsible for the transcription of messenger, ribosomal and transfer RNA. Much of the information regarding bacterial RNA polymerases and RNA synthesis has been obtained from studies of transcription in E. coli. The E. coli RNA polymerase is a multi-subunit complex composed of a β and β' subunit (of approximately 150,000 and 160,000 kilodaltons, respectively), two 43 kilodalton α subunits and one to two omega subunits approximately 11 kd in size (Burgess, 1969; Berg et al., 1971). The core polymerase catalyzes synthesis of transcripts but cannot accurately initiate transcription on natural DNA templates (Burgess et al., 1969; Sugiura et al., 1969; Travers and Burgess, 1969; Bautz and Bautz, 1971). An additional 70,000 dalton sigma subunit which is associated with the core enzyme is essential for specific transcription from many E. coli promoter sites. Recently, Grossman et al. (1984) demonstrated that the hptR gene product, which is responsible for inducing a number of proteins during the heat shock response (Neidhardt et al., 1984), also functions as a sigma factor to direct transcription from promoters that are not recognized by the sigma-70 enzyme.

The transcription process involves several sequential steps at which RNA synthesis can be controlled: promoter binding and initiation of RNA synthesis, elongation and

termination. In prokaryotes the sequence of events involved in the initiation of transcription plays an important role in the regulation of gene expression. The mechanism of promoter binding, the subsequent formation of initiation complexes and the regulation of these events have therefore been studied intensively in E. coli.

The current model for transcription initiation has evolved from the relatively simple scheme, suggested by the work of Walter et al . (1967), involving: 1) a binding reaction in which the RNA polymerase interacted specifically (and reversibly) in a "closed" complex with the DNA, 2) a melting reaction whereby the enzyme effected a localized unwinding of the DNA at the binding site to form an "open" complex and 3) RNA synthesis from the complex formed in step 2.

Hinkle and Chamberlin (1972b) investigated the kinetics of formation of phage T7 DNA/RNA polymerase complexes using a membrane filter binding assay in which DNA that has formed a stable association with RNA polymerase can be detected by its retention on nitrocellulose membrane filters. Their data showed that the binding reaction followed first-order kinetics, since the rate of the reaction was dependent only on the concentration of the RNA polymerase. They also observed that the rate of complex formation was slower than would be expected for a reaction which depended upon the diffusion-controlled interaction of the components. It was therefore hypothesized that the rate-limiting step in RNA

synthesis was the binding and subsequent release of polymerase from nonspecific sites on the DNA, prior to location of the promoter site. The model for the transcription initiation pathway was thus modified, as follows, to include a preliminary step in which the enzyme associates with non-specific sites on the DNA:

$$E+D_A \rightleftharpoons E\text{-}D_A \rightleftharpoons E \rightleftharpoons E\text{-}P_c \rightleftharpoons E\text{-}P_o$$

where $E\text{-}D_A$ represents the non-specific enzyme/DNA complex, and $E\text{-}P_c$ and $E\text{-}P_o$ are the specific closed and open promoter complexes hypothesized by Walter et al. (1967).

While there is considerable evidence to support the existence of promoter-specific closed and open complexes (for reviews see Chamberlin, 1974; von Hippel et al., 1982; McClure, 1985) there is at the present time no evidence that transient interactions of the polymerase with non-promoter DNA impedes promoter location (McClure, 1985). Stefano and Gralla (1979) observed that the formation of stable polymerase/DNA complexes on a restriction fragment which carried the lacUV5 promoter (measured in a filter binding assay, as described above) proceeded with a half-time of approximately 0.2 minutes. However, once formed, polymerase/lacUV5 promoter complexes did not rapidly synthesize RNA upon the addition of nucleoside triphosphates since the half-time for initiation from pre-formed complexes was approximately 1 minute. These data suggested that the initiation of RNA synthesis was rate-limited at a step subsequent to the formation of promoter-specific complexes.

Stefano and Gralla (1980) further investigated the formation of complexes with several mutant lac promoters. The reaction rates at the different promoters showed a first order dependence on the polymerase concentration and the rate varied by up to two orders of magnitude between promoters. A model was therefore proposed, in which the formation of complexes at the promoter proceeded through a kinetically unstable intermediate (equivalent to a closed complex). The rate at which functional complexes were formed would depend upon the stability of the intermediate and the rate at which it was converted to a functional complex. The term functional refers here to the ability of the complexes to directly initiate transcription. Operationally, functional complexes can be considered to be open complexes. McClure (1980) independently demonstrated that the binding of enzyme to promoters and subsequent steps could be separately quantitated.

Studies by Chamberlin and his colleagues (Kadesch et al., 1982; Rosenberg et al., 1982) have shown that template association and the formation of open complexes at the phage T7 A1 promoter are insensitive to changes in temperature (between 25 and 37°C) and NaCl concentration, suggesting that the rate-limiting step in the reaction occurs prior to open complex formation (ie. is not a DNA melting reaction). Based on this information, in addition to the work of Stefano and Gralla (1979, 1980) and McClure (1980), Kadesch et al. (1982) postulated the existence of a "pre-closed"

promoter complex (possibly analogous to DNA/RNA polymerase complexes at non-promoter sites) which isomerizes to form a closed complex. Finally, recent work by Buc and McClure (1985) and Roe et al. (1984) has also suggested the presence of an intermediate, observed only at low temperatures or high ionic strengths, between the closed and open complexes.

Based on the above data, McClure (1985) has proposed the following model for complex formation:

$$E+P \rightleftharpoons E\text{-}P_c \rightleftharpoons E\text{-}P'_c \rightleftharpoons E\text{-}P_i \rightleftharpoons E\text{-}P_o$$

The complexes $E\text{-}P_c$, $E\text{-}P'_c$ and $E\text{-}P_i$ are all closed complexes in which the enzyme is associated specifically with the promoter but the DNA helix at the promoter site is not unwound. In the open complex $E\text{-}P_o$ the DNA strands at the promoter are separated. It is this complex which can immediately initiate RNA synthesis in the presence of ribonucleotides. The sequence of events which lead to the formation of promoter-specific, functional complexes is thus quite complex and it is conceivable that the efficiency of transcription can be changed by altering one, or several steps in the pathway.

Transcription from many E. coli promoters requires only the RNA polymerase holoenzyme ($\beta\beta'\alpha_2\sigma$). Promoter structure (as well as structure of the DNA which surrounds the promoter) must therefore influence the enzyme/DNA interactions that determine the rate at which transcription is initiated from those promoters (see Rosenberg and Court, 1979; Hawley and McClure, 1983 for reviews).

E. coli promoters have two regions of homology centred approximately 35 and 10 basepairs upstream of the transcription start site. The consensus sequences at these sites are TTGACA and TATAAT, respectively (Rosenberg and Court, 1979; Siebenlist *et al.*, 1980). Mutations which alter promoter sequences such that the sequence more closely resembles the consensus sequence have been shown to enhance transcription. The *lac* operon promoter for example, which is only weakly transcribed in vivo in the absence of the CAP/cyclic AMP complex, has the -10 sequence TATGTT (Dickson *et al.*, 1975, 1977). The mutant *lac*UV5 promoter is altered at two sites in the -10 region such that the sequence is changed to the TATAAT consensus sequence (Gilbert, 1976). This promoter is able to direct RNA synthesis, *in vivo* and *in vitro*, in the absence of the CAP protein (Silverstone *et al.*, 1970; Gilbert, 1976; Stefano and Gralla, 1979).

The rates of complex formation at the *lac*UV5 and several other mutant lac promoters which differ by single base pair changes (Stefano and Gralla, 1980, 1982) have been shown to vary considerably. The differences in the reaction rates could be attributed solely to the differences in promoter structure since all of the promoters were contained on identical fragments of DNA. Alterations in promoter sequences can therefore lead to profound changes in transcription capacity.

Transcription in *Bacillus subtilis* has several features in common with *E. coli*, but there are several significant

differences as well. The B. subtilis RNA polymerase has a core assembly, similar to the E. coli core, that consists of a β and β′ subunit (approximately 140 and 130 kd, respectively), two 43 kilodalton alpha subunits and two small (11 and 9.5 kilodalton) omega peptides which are associated with the core but are of uncertain function (see Doi, 1982 for review). Apart from size, the core subunits differ in other respects from the E. coli subunits. For example, the sensitivity of the enzyme to the inhibitors streptolydigin (which inhibits elongation) and rifampicin (an initiation inhibitor) are determined by the beta subunit of the E. coli enzyme (Rabussay and Zillig, 1969; Iwakura et al., 1973). In B. subtilis the beta and beta′ subunits are responsible for rifampicin and streptolydigin sensitivity, respectively (Halling et al., 1977, 1978). The functions of these subunits are therefore likely to be different from the analogous E. coli subunits.

Bacillus subtilis has a number of sigma factors that determine the transcriptional specificity of the core enzyme, several of which are necessary for the transcription of genes involved in the process of sporulation (see Losick and Pero, 1981; Doi, 1982 for reviews). In vegetative cells the predominant form of RNA polymerase is associated with a sigma factor (formerly designated sigma-55) of only 43 kilodaltons. Sigma-43 is considerably smaller than the 70 kd E. coli sigma factor but has several regions that are homologous with the E. coli subunit (Wong and Doi, 1982;

Gitt et al., 1985). The sigma-43 RNA polymerase directs transcription from promoters that have the same consensus sequence as the E. coli promoters (Moran et al., 1982). It has been observed that some promoters which are transcribed by the E. coli enzyme are not transcribed very efficiently by the Bacillus polymerase (Davison et al., 1979, 1980; Moran et al., 1982), suggesting that the Bacillus enzyme might have more stringent requirements for promoter structure than the E. coli enzyme.

A 21,000 dalton protein, first described by Pero et al. (1975), is also associated with the Bacillus subtilis sigma-43 RNA polymerase. This subunit, designated delta, has been shown to be distinct, both immunologically and by peptide mapping analysis, from sigma-43 (Wong and Doi, 1982).

Delta has no known counterpart in E. coli and its role in transcription has therefore been studied extensively. Core/delta has been shown to have very low activity in vitro with various phage, chromosomal or plasmid templates (Tjian et al., 1977; Williamson and Doi, 1978; Spiegelman et al., 1978) suggesting that delta does not confer initiation activity upon the enzyme. The protein nevertheless does influence transcriptional specificity. Pero and her colleagues found that specific in vitro transcription of phage SP01 middle genes required the host delta protein (Pero et al., 1975). Similar data were reported for transcription from SP82 DNA by phage-modified and host cell RNA polymerase (Spiegelman et al., 1978; Achberger and

Whiteley, 1981). The overall effect of delta, however, seems to be dependent upon the template. Dickel et al. (1980) observed that the activity of core/sigma-43 on Bacillus chromosomal DNA was very sensitive to inhibition by delta. In contrast, transcription from the Bacillus phage templates Øe and Ø29 was essentially unaffected by added delta.

Spiegelman et al. (1978) used a filter binding assay to study the effect of delta on the formation of enzyme/DNA complexes with SP82 DNA. They observed that in the presence of delta, less DNA was bound to the filters, at all concentrations of enzyme tested. Delta appeared to inhibit complex formation by destabilizing the enzyme/DNA complexes, presumably at non-specific sites on the DNA. More direct studies on complex formation by the sigma-43 RNA polymerase have employed a modified filter binding assay in which DNA restriction fragments that form non-filterable complexes with the enzyme are eluted from the filters and analyzed by gel electrophoresis. The results of such assays have demonstrated that delta restricts complex formation to phage ϕ29 DNA restriction fragments which carry promoters that are recognized by the sigma-43 enzyme (Dickel et al., 1980). Achberger and Whiteley (1981) have reported similar findings for complex formation on phage SP82 DNA.

It has been proposed that the Bacillus subtilis RNA polymerase requires both sigma and delta for efficient promoter recognition and transcription initiation

(Williamson and Doi, 1978; Dickel et al., 1980; Achberger and Whiteley, 1981); the functions which are carried out solely by the E. coli sigma factor are associated with either sigma-43 or delta in Bacillus. Achberger and Whiteley (1981) have further suggested that sigma is required for promoter recognition and initiation, while delta functions to reduce binding at non-specific sites on the DNA by destabilizing such non-specific complexes. Williamson and Doi (1978) have proposed a model in regard to the delta-sigma interactions which regulate promoter activity. The details of this model will be considered in the Discussion.

The effect of delta upon specific polymerase interactions with an early gene promoter of phage SP82 has been investigated by methylation protection and DNase I footprinting analysis (Achberger et al., 1982). These studies revealed that the presence of delta did not affect the region of DNA which was protected by the RNA polymerase but did cause a decrease in the number of contacts between the enzyme and promoter, in non-initiated complexes. Delta did not however, significantly alter the pattern of contacts formed in initiated complexes, suggesting that it acted prior to initiation. This latter result supported a previous report by Spiegelman and Whiteley (1979) that delta was released from enzyme/DNA complexes prior to initiation. It was also observed that 11 out of 15 contacts present in core/sigma non-initiated complexes were present in

formed contacts with the promoter that are normally seen only in initiated complexes. It was therefore proposed by Achberger *et al*. (1982) that delta altered the conformation of the RNA polymerase to prevent premature formation of initiation-specific contacts, resulting in less efficient complex formation at nonpromoters or weak promoters.

The mechanism of RNA synthesis initiation by the *Bacillus subtilis* RNA polymerase has not previously been investigated. The information about the effect of delta on transcription and polymerase/promoter interactions suggested that a kinetic approach could be used to analyze the mechanism by which delta affects transcription and promoter selectivity. The initial work presented in this thesis therefore focussed upon the development of an assay, analogous to those used in studies of *E. coli* transcription kinetics, which would be appropriate for the analysis of transcription initiation by the *B. subtilis* sigma-43 RNA polymerase. The other goal of this work was to use the assay to investigate the manner in which delta affected the kinetics of promoter-specific interactions.

MATERIALS AND METHODS

Phage and Plasmid DNA

Bacteriophage Ø29 was grown in B. subtilis SR22 or L15 (both the phage and the hosts were obtained from H.R. Whiteley, University of Washington) and phage was purified as described previously by Kawamura and Ito (1977). Purified phage were treated with 1% SDS, 20 mM EDTA and heated at 65°C for 10 minutes. The ruptured particles were then treated with 50 ug/ml Proteinase K for 2 hours at room temperature. The DNA was purified by phenol extraction and precipitated in the presence of 3% sodium acetate. The DNA was dissolved in sterile distilled water, dialyzed at 4°C overnight against 0.1xSSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). and stored over chloroform.

Plasmid bearing strains were grown as described by Dunn et al. (1979). Cleared lysates were prepared by the procedure of Clewell and Helinski (1972) with several modifications as described by Dobinson and Spiegelman (1985). Plasmid DNA was purified from the cleared lysate by centrifugation in CsCl/ethidium bromide. The isolated plasmid DNA was treated with butanol to remove the ethidium bromide, ethanol precipitated and redissolved in distilled water or 0.1xSSC. Small scale plasmid preparations, used to screen transformants, were made as described by Crosa and Falkow (1981).

Media

Bacillus subtilis was grown in M medium (Yehle and Doi, 1967). Plasmid bearing Escherichia coli strains were grown in M9 medium (Champe and Benzer, 1962) modified as follows: the glucose concentration was 0.25%, casamino acids were added to 0.2%, and $FeCl_3$ and $CaCl_2$ were added to a concentration of 0.01 mM and 0.1 mM respectively. The medium was supplemented with uridine (to 200 ug/ml), thymidine (to 10 ug/ml) and thiamine (to 20 ug/ml).

LB (Luria-Bertani) plates used in the cloning procedure were prepared as described by Maniatis et al. (1982).

Construction of p328-5

The plasmid p328-5 contains sequences from the Bacillus phage Ø29. Phage Ø29 was cleaved with HindIII and the 2.4 kb fragment (Band C) was isolated by electroelution from a 4% polyacrylamide gel. The vector, pBR322, was cut with HindIII, treated with calf intestinal phosphatase, and ligated to the 2.4 kb fragment. E. coli SF8 (obtained from R.C. Miller, University of British Columbia) was transformed with the ligation mixture and and spread onto LB plates containing 50 ug/ml ampicillin. Colonies from the ampicillin plates were picked and tested for growth on LB plates containing 20 ug/ml tetracycline. Several colonies which were resistant to ampicillin but sensitive to

tetracycline were screened for the presence of plasmids which were larger than the original vector. Plasmid p328-5 was shown to have the correct DNA sequence by restriction analysis and by RNA-DNA hybridization analysis (see Results).

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, alkaline phosphatase was supplied by Boehringer/Mannheim and all were used according to the suppliers' recommendations. Manipulation of plasmid DNA, gel electrophoresis and cloning procedures were all carried out according to Maniatis et al.(1982).

DNA-RNA Hybridization Analysis

Hybridization analysis was used to confirm that the major in vitro transcript synthesized from p328-5 was initiated from the ∅29 promoter. The [α-^{32}P]UTP labelled products of a standard transcription reaction were separated by electrophoresis through a 7 M urea, 8% polyacrylamide gel (40% acrylamide:1.38% bisacrylamide) and the run-off transcript eluted from the gel as described by Maniatis et al. (1982), except that the elution buffer was as described by Maxam and Gilbert (1977).

Plasmid and ∅29 DNA restriction fragments were separated by electrophoresis through 0.7% agarose gels and transferred to a nitrocellulose membrane filter as previously described by Dunn and Sambrook (1980) except that

the neutralization buffer contained 1 M Tris-HCl pH 7.4, 1.5 M NaCl. The DNA was baked onto the filters at 65°C, overnight. The nitrocellulose membrane was pre-soaked for 3 hours at 65°C in 6xSSC, 0.02% BSA. The membrane was then transferred to hybridization buffer containing 6xSSC, 0.02% BSA, 1 mM EDTA and 0.5% SDS. The labelled RNA was added, and hybridization carried out for 18 to 24 hours at 65°C. The membrane was washed at 65°C in 2xSSC, 0.5% SDS for 40 minutes, then in 2xSSC for 40 minutes. Hybridization was detected by autoradiography using 3M Hi Lite film (Kodak) at -70°C.

RNA Polymerase Purification

Purification of Holoenzyme (core/sigma/delta). RNA Polymerase (sigma-43) was purified from Bacillus subtilis 168 (Iowa Grain Processing Corp.) as previously described by Spiegelman et al. (1978) by: 1) PEG/dextran phase partitioning, 2) chromatography on Bio-Gel A 1.5m, 3) chromatography over DNA-cellulose, 4) sedimentation through a glycerol gradient. The above procedure was modified in several ways. 1) The DNA-cellulose was prepared as described by Litman (1968) with calf thymus or salmon sperm DNA. 2) The glycerol gradient was 15 to 30% and contained 0.5 M NaCl. 3) The fractions from the fastest sedimenting portion of the the glycerol gradient enzyme activity band were pooled and chromatographed over heparin-

agarose (Sigma Chemical Co.) or heparin-sepharose (prepared with Sepharose 4B (Pharmacia) as described by Davison et al., 1979) to remove a contaminating DNase activity. The gradient fractions were diluted with buffer to a conductivity equal to that of buffer containing 0.1 M NaCl (conductivity was measured with a Radiometer CDM 2f conductivity meter). The diluted sample was applied to the heparin-agarose column which had been equilibrated with buffer containing 0.1 M NaCl and the column was washed with buffer containing 0.1 M NaCl, then with 8 to 10 volumes of 0.35 M NaCl-containing buffer. The enzyme was eluted in a single step with buffer containing 0.6 M NaCl.

All the buffers for the above chromatography steps, as well as for those procedures described below for the isolation of core/sigma and delta, contained 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 50 ug/ml PMSF, 20 mM 2-mercaptoethanol and 10% glycerol. It should be noted here that the enzyme seemed to be particularly sensitive to the type of glycerol used in the buffers. In particular, when Sigma grade glycerol (Sigma Chemical Co.) was used during the isolation procedure the purified enzyme was very unstable and lost 50 to 90% of its activity in one to two weeks. Therefore, analytical grade glycerol (obtained from BDH Chemicals) was used in the purification of both enzyme and delta. The enzyme was stored at -12°C in buffer, described above, containing 30% glycerol and 0.46 M NaCl.

Enzyme activity was assayed as described by Spiegelman and Whiteley (1974), with several modifications. Assays were carried out in 0.04 M Tris-HCl pH 7.9, 0.02 M $MgCl_2$, 0.05 M NaCl, with 0.8 mM GTP, ATP and CTP, 8 uM UTP and 0.25 uCi [^{3}H]UTP using ø29 DNA as the template. The reaction mixtures were precipitated with TCA in the presence of 100 ug/ml yeast RNA (Type II-S from Torula yeast, Sigma Chemical Co.). Unless otherwise stated enzyme concentrations in the text are in terms of protein concentration (determined by a Lowry assay, as modified by Sandermann and Strominger, 1972) using BSA (Fraction V, Sigma Chemical Co.) as the standard.

The proportion of active enzyme in a preparation was estimated on the basis of the sigma content in the preparation. The enzyme was electrophoresed on 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 hours 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 in 7.5% acetic acid and scanned with a Quick Scan densitometer (Helena Instruments). The areas under the peaks which corresponded to the sigma, delta and alpha subunits were determined by weight. The sigma:alpha ratio was calculated using an estimated molecular weight of 45,000 daltons for the alpha

subunit (Doi, 1982) and a molecular weight of 43,000 daltons for the sigma subunit (Gitt et al., 1985). The amount of alpha subunit, which is a component of the core enzyme, was considered to be representative of the amount of core in the enzyme preparation. It should be noted that the amount of active enzyme in the preparations may be overestimated by this method since it was not possible to determine if all of the enzyme which was associated with sigma was in fact active.

Isolation of Core/sigma. Holoenzyme (core/sigma/delta) was depleted of delta by DNA-cellulose chromatography of glycerol gradient purified enzyme (Achberger and Whiteley, 1981) or by DEAE-Sephadex chromatography (Spiegelman et al., 1978) of heparin-agarose purified enzyme.

The DNA-cellulose purification was carried out as follows: glycerol gradient fractions were combined and diluted as described above and applied to a DNA-cellulose column (2.5 cm x 8 cm). Elution with a linear gradient of NaCl (0.1 M to 0.6 M) separated core/sigma from delta, the core/sigma activity eluting between 0.35 and 0.55 M NaCl. The enzyme fraction was then concentrated in an Amicon apparatus prior to chromatography over a heparin-agarose column (described above).

Holoenzyme which had been purified through the heparin-agarose step was diluted with buffer to give a conductivity

equivalent to that of buffer containing 0.1 M NaCl and applied to a DEAE-Sephadex column which had been equilibrated with buffer containing 0.1 M NaCl. The column was then washed with buffer containing 0.1 M NaCl, and core/sigma was eluted in a single step with buffer containing 0.32 M NaCl.

The amount of delta present in the core/sigma and holoenzyme preparations was determined by the method described above for determing the sigma content of the enzyme. Core/sigma was reconstituted with delta by incubating delta with core/sigma for two minutes at 37°C prior to adding the enzyme to the transcription reactions. The pre-incubation was not critical however, since the results of the experiments were not affected if that step was omitted.

Purification of the Delta Subunit

Delta was purified from pooled delta-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 to 60 minutes) and centrifuged at low speed to remove the resulting precipitate. The supernatant contained delta, which is heat stable (M. Hilton, personal communication), that could be further purified by chromatography over DEAE-Sephadex (Spiegelman et al., 1978).

The amount of delta 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, scanned and the areas under the peaks which corresponded to BSA and delta were quantitated as described above. The weights of the BSA peaks were used to generate a standard curve and the amount of delta present in the delta preparations was estimated by comparison of the delta peak weight with the standard curve. The molar concentration of delta was subsequently calculated using an estimated molecular weight of 21,000 daltons for the delta subunit (Doi, 1982).

In vitro Transcription Assays

Run-off transcriptions were done in 40 or 100 ul volumes in buffer containing 40 mM Tris-HCl pH 7.9, 20 mM $MgCl_2$, 1 mM EDTA, 48.5 mM NaCl and 3% glycerol. The ATP, GTP and CTP concentrations were 400 uM and the UTP concentration was 10 uM. All nucleotides were obtained from Sigma Chemical Co.

The standard (multiple round) transcription reactions were initiated by the addition of RNA polymerase to a solution containing DNA, ATP, GTP, CTP and UTP which had been prewarmed for 2 minutes at 37°C. Transcription was carried out for 10 to 15 minutes at 37°C and the reactions were stopped by the addition of EDTA (final concentration

25 mM) or electrophoresis sample buffer (10 M urea, 0.1 M Tris-Borate, 2 mM EDTA) and chilled on ice.

Unless otherwise stated the kinetic (productive transcription) assays were carried out as follows: a solution containing DNA, ATP and GTP was prewarmed for 2 minutes at 37°C, RNA polymerase (which had also been prewarmed) was added and at various times after the addition of the enzyme, samples were removed and added to a mixture of heparin (Grade I, Sigma Chemical Co.), CTP and [α-^{32}P]UTP (1 to 5 uCi/reaction; Amersham, 410 Ci/mmol) to allow elongation of initiated transcripts. Heparin was used at a final concentration of 5 ug/ml, to prevent multiple initiation events at one promoter. The elongation reaction was continued for 10 minutes, then the reactions were stopped with EDTA or electrophoresis sample buffer and chilled on ice or frozen at -20°C. Aliquots of the transcription reactions were electrophoresed through 8% acrylamide, 7M urea gels. The run-off transcripts were localized by autoradiography, cut from the gel and the radioactivity in the gel slices quantitated by Cerenkov counting.

The sequences of the 029 promoter-specific run-off RNAs and number of U residues in them was known (66 in the A2 transcript and 22 in the G2 transcript). The number of transcripts synthesized from the promoters could therefore

be calculated from the amount of radioactivity which was incorporated into the run-off transcripts.

RESULTS

CLONING AND LOCALIZATION OF THE ϕ29 A2 PROMOTER

The bacteriophage ϕ29, a double stranded DNA phage which is able to infect several Bacillus species (Hemphill and Whiteley, 1975; Geiduschek and Ito, 1982), is one of the most extensively characterized Bacillus genetic systems (for review see Geiduschek and Ito, 1982). Transcription of the ϕ29 genome has been studied in detail. Ribonucleic acid which is synthesized during the lytic cycle can be divided temporally into two classes; early RNA is transcribed from the light strand of the phage DNA throughout the phage infection, whereas late RNA is transcribed from the heavy strand (Mosharaffa et al., 1970; Loskutoff et al.,1973; Schachtele et al.,1973).

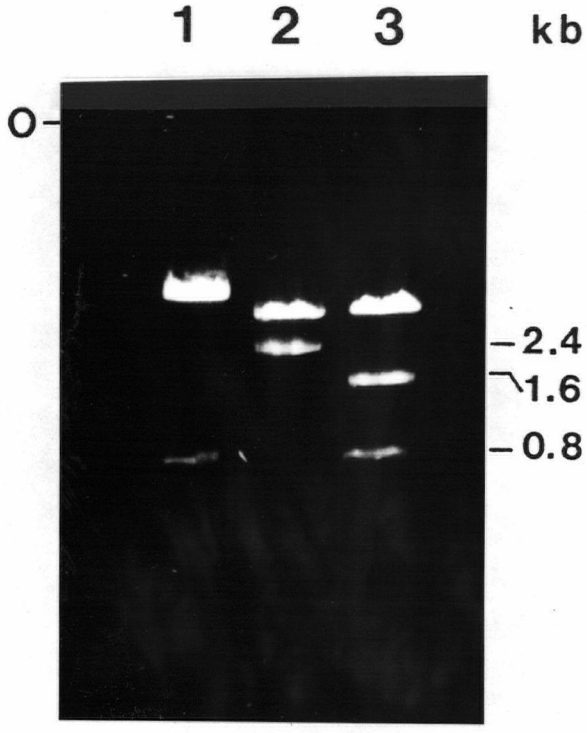
In vitro transcription by the B. subtilis RNA polymerase can only be initiated from promoters for early RNA. These promoters appear to be recognized by the predominant sigma-43 associated Bacillus polymerase (Duffy and Geiduschek, 1976). Detailed in vivo and in vitro transcription maps of ϕ29 DNA have been constructed (Loskutoff et al., 1973; Inciarte et al., 1976; Kawamura and Ito, 1977; Davison et al., 1980) and the approximate locations of the early promoters have been determined (Sogo et al., 1979; Davison et al., 1980; Dickel et al ., 1980). Several promoter sites have also been tentatively located by

nucleotide sequence analysis of the ∅29 genome (Yoshikawa et al.,1981; Yoshikawa and Ito, 1982).

One of the ∅29 early promoters, which had been localized to the ∅29 HindIII-C restriction endonuclease fragment (Sogo et al., 1979; Davison et al., 1979, 1980), was cloned into pBR322, characterized and used in the transcription studies described below.

Plasmid p328-5 Carries the HindIII-C Restriction Fragment from ∅29. The 2.4 kb HindIII-C fragment of ∅29 contains a single EcoRI restriction site near one end of the fragment (Ito, 1978). Restriction analysis with HindIII and EcoRI (see Figure 1) was therefore carried out on p328-5 to verify that the HindIII-C fragment had been cloned into pBR322 and to determine the orientation of the cloned insert. Treating the plasmid p328-5 with the restriction enzyme HindIII (Figures 1 and 3, lane 2) yielded two fragments. The small fragment migrated at the position of ∅29 HindIII-C fragment (see Figure 3), indicating that the HindIII-C fragment had been cloned, intact, into the vector plasmid. When p328-5 was treated with EcoRI (Figure 1, lane 1) a small band of approximately 0.8 kb (estimated by comparison with ∅29 HindIII restriction fragments) was released. Since the cloned fragment had a single, asymmetric EcoRI site and the vector (pBR322) also had one EcoRI site the orientation of the cloned DNA must be as shown in Figure 2.

Figure 1. Restriction analysis of the plasmid p328-5. The plasmid was digested with EcoRI (lane 1), HindIII (lane 2) or EcoRI and HindIII (lane 3), run on a 1.0% agarose gel and stained with ethidium bromide. The sizes of the cloned DNA fragments and the origin of electrophoresis (O) are indicated.


1
2
3
kb
O
2.4
1.6
0.8

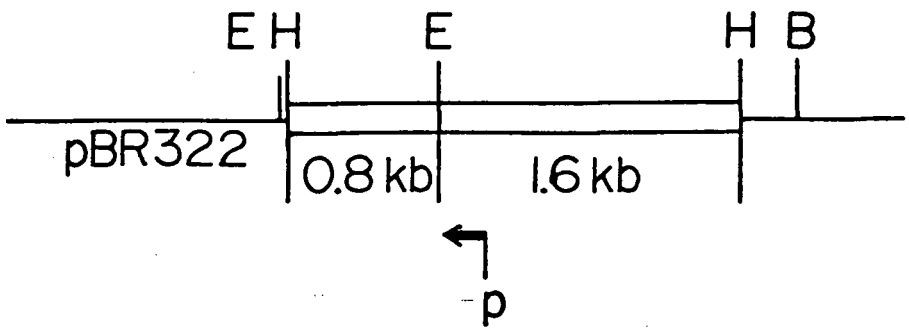
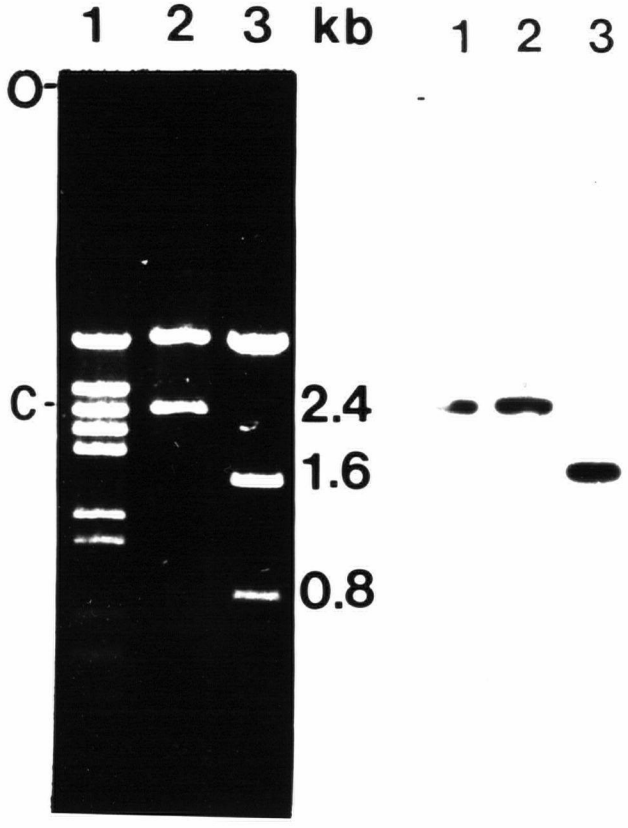


Figure 2. Plasmid p328-5. A 2.4 kb fragment from 029 DNA, delineated by HindIII restriction sites, was isolated and cloned into the HindIII site of pBR322. Restriction sites are designated as follows: E-EcoRI, H-HindIII, B-BamHI. The position and orientation of the promoter (p) are indicated.

Location of the ϕ29 Promoter on p328-5. The location of a promoter on the HindIII-C fragment of ϕ29 had been predicted from the work of Davison et al. (1980) and Sogo et al. (1979). When p328-5 was treated with EcoRI and transcribed in vitro (in a standard assay, as described in Materials and Methods) with the Bacillus subtilis sigma-43 RNA polymerase a discrete RNA transcript was observed (for example see Figures 12a and b). The size of the RNA was approximately 200 bases long as estimated by comparison to the xylene cyanol tracking dye.

The transcript was hybridized to restriction fragments of p328-5 and ϕ29 DNA which had been electrophoresed through an agarose gel and bound to nitrocellulose, as described in Materials and Methods. The in vitro RNA hybridized exclusively to the 1.6 kb HindIII-EcoRI fragment from p328-5 and to band C from a HindIII digest of ϕ29 DNA (Figure 3). The transcript synthesized from EcoRI-treated p328-5 thus had no homology with other ϕ29 sequences or pBR322. The size of the RNA suggested that either the promoter was located near the EcoRI site on the 1.6 kb EcoRI-HindIII fragment with transcription directed towards the EcoRI site as shown in Figure 2 or that transcription initiated and terminated within the 1.6 kb fragment. The former conclusion was consistent with previous data (Loskutoff et al., 1973; Schactele et al., 1973; Davison et al., 1979, 1980). The promoter (A2) was subsequently sequenced and the in vivo and in vitro transcription initiation sites

Figure 3. Hybridization of p328-5 transcripts to p328-5 and ϕ29 DNA restriction fragments. (Left) Ethidium bromide stained 0.7% agarose gel prior to blotting. Lane 1, 029 DNA digested with HindIII. Lane 2, p328-5 digested with HindIII. Lane 3, p328-5 digested with EcoRI and HindIII. Sizes of the cloned DNA fragments (estimated by comparison with the 029 HindIII fragments) are indicated. (Right) Autoradiogram of the nitrocellulose blot after hybridization to the p328-5 transcript. The lanes are the same as in (Left). The origin of electrophoresis (O) and the position of the HindIII-C band are indicated.


1 2 3 kb
1 2 3
O
C
2.4
1.6
0.8

determined by S1 nuclease mapping (Dobinson and Spiegelman, 1985).

PURIFICATION OF THE RNA POLYMERASE AND DELTA FACTOR

Holoenzyme which had been purified through glycerol gradients (as described in Materials and Methods) could be used for qualitative in vitro transcriptions. However, quantitative analysis of transcription was hampered due to the presence of a contaminating DNase activity in the enzyme preparations.

The glycerol gradient enzyme, pooled from the peak activity fractions, was further purified over heparin-agarose as described in Materials and Methods in an attempt to remove the DNase activity. The autoradiogram presented in Figure 4 shows the results of one experiment in which glycerol gradient-purified enzyme and enzyme which had been subsequently purified by heparin-agarose chromatography were tested for DNase activity. The two enzymes were incubated in the presence of ATP, GTP and CTP under the same conditions used for in vitro transcriptions, with a 273 base HindIII restriction fragment, from phage Ø29 DNA, which had been labelled at the HindIII site with [α^{32}P]-dATP. Each reaction contained approximately 4,000 cpm DNA. The reactions were precipitated with ethanol prior to electrophoresis through a 5% polyacrylamide, nondenaturing gel and the bands on the gel were quantitated by Cerenkov counting. After incubating the glycerol gradient enzyme

Figure 4. Assay for DNase activity in the RNA polymerase preparations. A ^{32}P end-labelled restriction fragment of Ø29 DNA was incubated with glycerol gradient purified enzyme (lane 1), or Heparin-agarose purified enzyme (lane 2) under conditions that were used for *in vitro* transcription reactions. The reaction mixtures were electrophoresed through a nondenaturing polyacrylamide gel and the remaining radioactivity in the DNA bands quantitated as described in the text. The lower band corresponds to the Ø29 restriction fragment isolated prior to end-labelling.


1
2

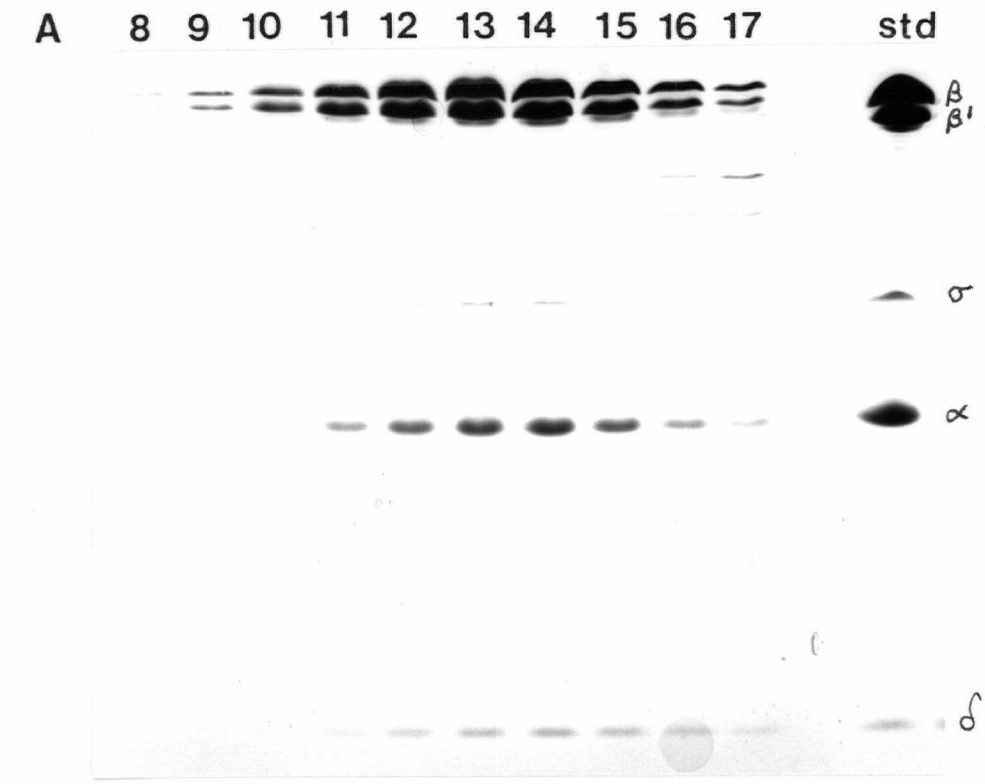
with the DNA for 10 minutes the DNA was no longer detectable on the gel (lane 1). The amount of radioactivity recovered from the gel at the position where the DNA would have banded was 264 cpm. When the heparin-agarose purified enzyme was incubated with the DNA the amount of radioactivity in the gel slices corresponding to the DNA bands was 4,024 cpm (lane 2). Although the DNA was isolated as a single band prior to labelling, two bands were detected on the gel subsequent to incubation with the enzyme. The reason for the anomolous electrophoresis of the DNA is not clear.

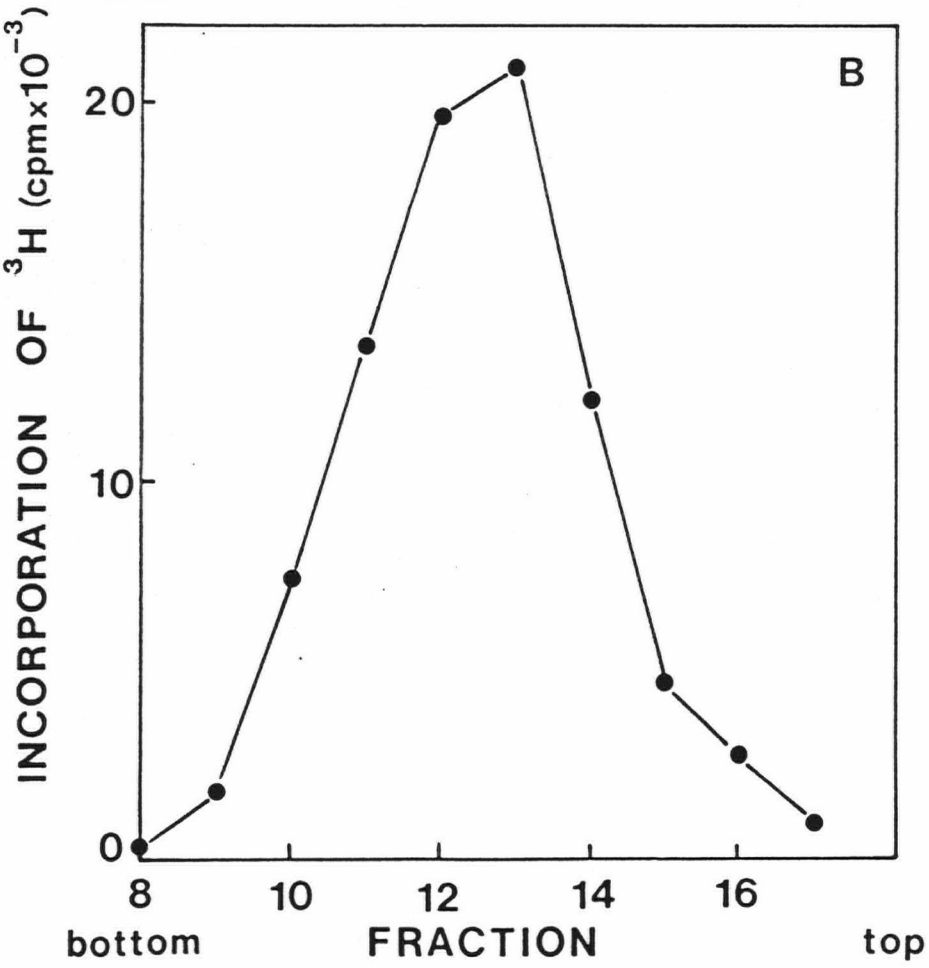
During the course of this work it was observed that when the entire enzyme band from the glycerol gradient was combined and purified over heparin-agarose, the resulting enzyme appeared to lose activity when the initiation reaction of the productive transcription assay (described above in Materials and Methods) was carried out for 10 to 20 minutes. Although the DNase activity had been removed from the enzyme there appeared to be some other inhibitory activity in the preparation. Another observation was that the activity profile from the glycerol gradient did not correlate well with the amount of enzyme in each fraction (see Figure 5). Some fractions from the leading edge of the band had more activity, but less protein, than fractions from the latter part of the band. Although this discrepancy could in part be accounted for by the presence of the DNase in some of the fractions it was possible that there was some other inhibitor in the latter fractions as well.

In an attempt to solve the problem the fractions of enzyme from the leading edge of the band were taken for subsequent purification. For example, fractions 10-13 from the glycerol gradient shown in Figure 5 were combined and purified over heparin-agarose, as described in Materials and Methods. The enzyme that was loaded onto the column had a total activity of $2x10^7$ cpm (^3H incorporated into TCA precipitable material in an in vitro transcription assay, as described in Materials and Methods) and the enzyme that was eluted from the column by buffer containing 0.6 M NaCl had an activity of 1.5×10^7cpm. The recovery (75%) was typical of the recovery from this stage of the purification, which ranged between 60 and 75%. The resulting enzyme retained activity after a 10 minute incubation in the presence of DNA, ATP and GTP and could be used in the productive transcription assays and tau plot analysis. At the present time it is not clear why there should be a difference between enzyme which is in the different fractions of the glycerol gradient peak.

Delta was removed from holoenzyme (core/sigma/delta) by purification over DNA-cellulose or by DEAE-sephadex chromatography as described in Materials and Methods. The two enzymes were not detectably different since both enzymes responded in the same manner in a productive transcription assay when reconstituted with delta (for an example of the response see Figures 13 and 14). Neither purification procedure removed all the delta peptide from holoenzyme. In

Figure 5. (A) SDS-polyacrylamide gel electrophoresis of purified Bacillus subtilis RNA polymerase. Fractions 8 to 17 from a glycerol gradient were electrophoresed through a 14-20% gradient gel, as described in Materials and Methods. Enzyme purified from the glycerol gradient fractions 10 to 13, by heparin-agarose chromatography is shown in the lane designated std. The positions of the subunits ($\beta, \beta', \alpha, \sigma, \delta$) are indicated. (B) Activity profile from the glycerol gradient fractions shown in (A). The enzyme activity in the glycerol gradient fractions was assayed using [^{3}H]UTP as described in Materials and Methods.


A
8
9
10
11
12
13
14
15
16
17
std
β
β'
σ
α
δ


B
INCORPORATION OF ^{3}H (cpm×10^{-3})
20
10
0
8
10
12
14
16
bottom
FRACTION
top

comparison to holoenzyme the amount of delta present in the core/sigma preparations was reduced by approximately 50% (for example see Table III). For the sake of clarity, enzyme which has been depleted of delta will be subsequently designated core/sigma.

When delta was purified from glycerol gradient fractions by DEAE-sephadex chromatography a protein with an apparent molecular weight of 55 to 66 kilodaltons was often detected by SDS-polyacrylamide gel electrophoresis of the column fractions which contained delta. This band did not consistently migrate to the same position in the gels in relation to the RNA polymerase subunits and the amount of contaminant relative to the delta band also varied from day to day. The contaminant was not visible on nondenaturing gels and it was concluded that the high molecular weight protein was probably an aggregate of delta. No other contaminating proteins were evident in the delta preparations (see Figure 6) and as shown in Table I and Figure 7 delta was free of RNase, DNase and proteolytic activity.

CHARACTERIZATION OF THE CONDITIONS FOR THE *IN VITRO* SINGLE ROUND TRANSCRIPTION ASSAY

Studies of the *E. coli* RNA polymerase have shown that, in general, enzyme which associates with a promoter proceeds through a "closed " state to an "open" complex state which

Figure 6. SDS-polyacrylamide gel electrophoresis of purified delta. The samples were electrophoresed through a 14-20% gradient gel, as described in Materials and Methods. Lane 1, Heparin-agarose purified enzyme. Lane 2, purified delta preparation. The positions of the subunits are indicated.


1
2
β
β'
γ
α
δ

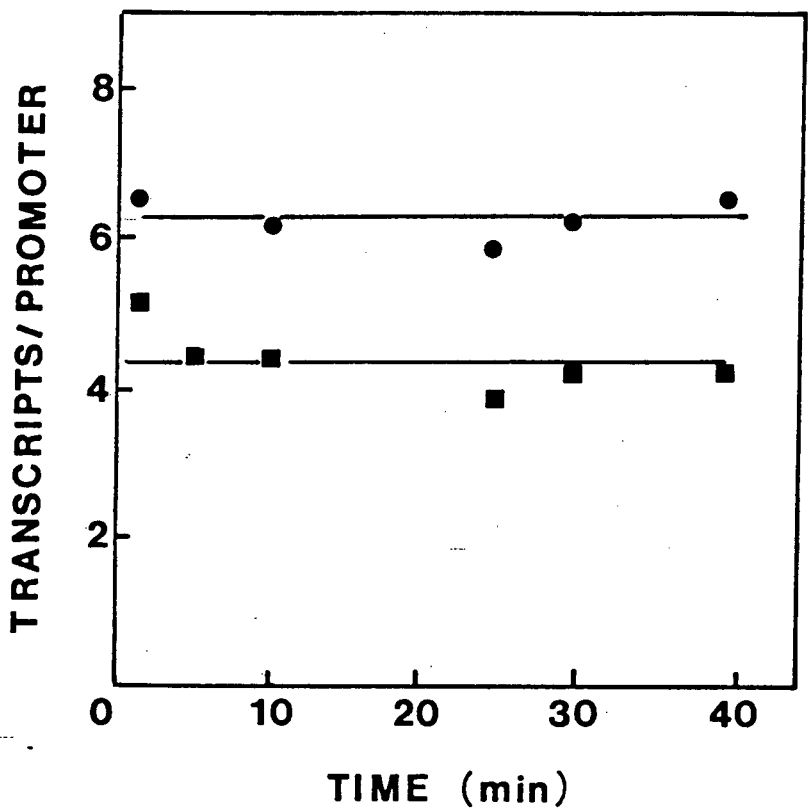
TABLE I

Assay for RNase and DNase activity in the delta preparation

Delta	Average cpm ($x10^{-5}$) DNA	Average cpm ($x10^{-4}$) RNA
+	1.75 ± 0.05	3.01 ± 0.34
-	1.65 ± 0.05	2.28 ± 0.66

^{32}P-labelled DNA or *in vitro* RNA synthesized from EcoRI-treated p328-5 was incubated at 37°C for 10 minutes with delta (13-15 pmol) or enzyme storage buffer (described in Materials and Methods) under the same conditions used for in vitro transcriptions. The RNA or DNA samples were then electrophoresed on polyacrylamide gels, and the bands in the gel were excised and quantitated for radioactivity by Cerenkov counting. The values are averages of triplicate DNA and duplicate RNA samples ± the standard deviation.

Figure 7. Assay for proteolytic activity in the delta preparation. Core/σ was incubated at 37^{0}C in the presence (■) or absence (●) of delta for 1 to 40 minutes before being added to a standard transcription reaction. The transcriptions were initiated by the addition of 80 nM enzyme to the template DNA (at 1.7 nM) and ribonucleotides and carried out for 10 minutes at 37^{0}C. Delta was added to core/sigma at a ratio of 0.5 mol. delta:1 mol. enzyme.


TRANSCRIPTS/ PROMOTER
8
6
4
2
0
10
20
30
40
TIME (min)

is stable and relatively resistant to inactivation by heparin (Walter et al., 1967; Chamberlin, 1974; McClure, 1980; Stefano and Gralla, 1980). Enzyme which is free in solution, bound at non-specific sites on the DNA or in "closed" complexes at promoters is inactivated by heparin.

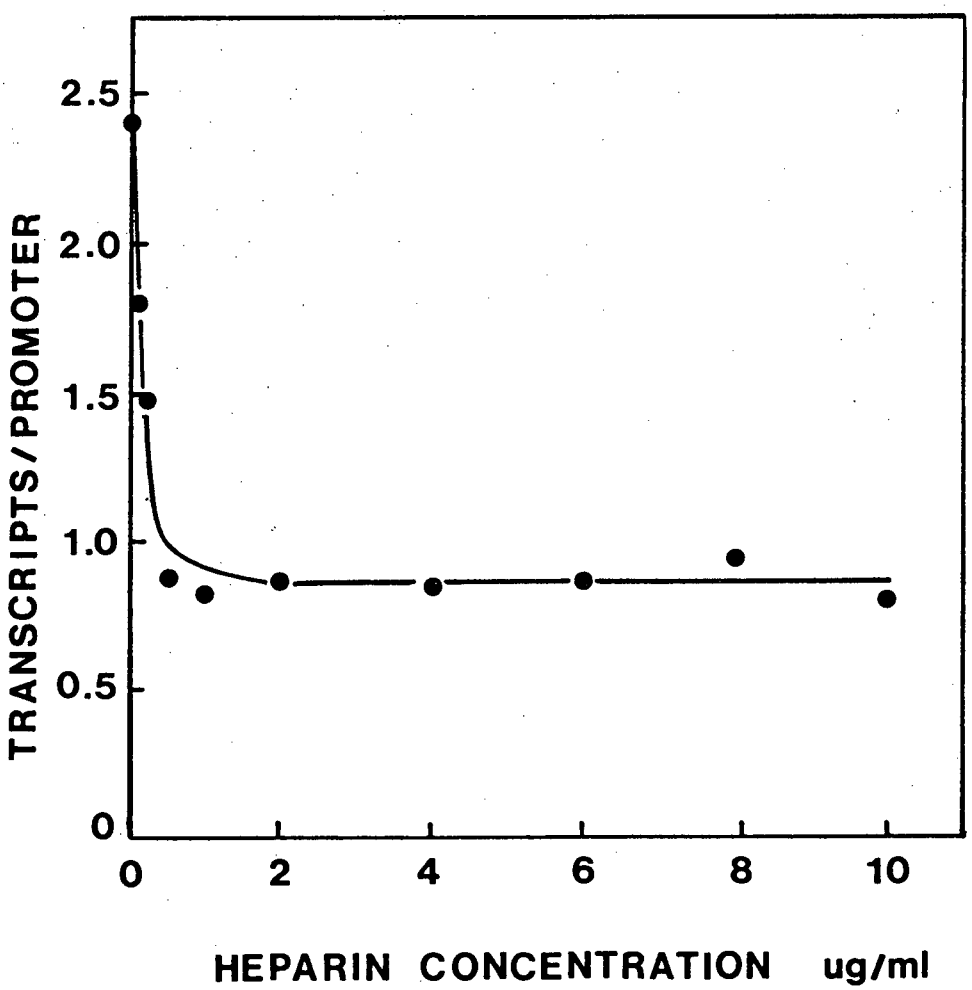
In the productive transcription rate assay developed by Stefano and Gralla (1980) to study E. coli RNA polymerase interactions at mutant lac promoters, RNA polymerase and promoter-containing restriction fragments are allowed to form complexes for varying lengths of time before nucleoside triphosphates and heparin are added to the reaction. Enzyme which is in an open complex at the time of heparin addition will proceed to synthesize a run-off transcript which terminates at the end of the restriction fragment. The run-off transcripts are the products of a single round of initiation and the incorporation of radioactivity into the transcripts can be used to quantitate the number of open complexes formed prior to the addition of heparin. The kinetics of formation of such complexes can therefore be studied using a single round transcription assay.

The productive transcription rate assay was modified in this work to investigate the initiation of transcription at the ø29 A2 and G2 promoters by the B. subtilis RNA polymerase. The conditions for the assay differ from those of the Stefano and Gralla method in three ways. First, instead of using promoter-containing restriction fragments, run-off transcripts were synthesized from plasmid which had

been cleaved at a restriction site downstream from the promoter of interest. There was thus more non-specific DNA in my assays than in the assays carried out by Stefano and Gralla (1980, 1982). Secondly, preliminary experiments with the ϕ29 A2 promoter showed that the simultaneous addition of heparin and ribonucleotides to promoter/polymerase binary complexes resulted in a very low level of transcription (see Figures 12 a, b). These data suggested that the RNA polymerase did not form heparin-resistant, non-initiated complexes with the A2 promoter. Since the nucleotide sequence of the transcript initiated from the A2 promoter site begins with pppAGA (Dobinson and Spiegelman, 1985), ATP and GTP were included in the reactions to allow the enzyme to form initiated complexes at the promoter. The modified composite rate assay used in this work thus measured the rate of formation of heparin-resistant, initiated complexes, rather than the formation of heparin-resistant, non-initiated complexes. Finally, the concentration of heparin used in the assays was 5 ug/ml, as opposed to 100 ug/ml used by Stefano and Gralla (see Figure 8).

The effect of heparin concentration on transcription from the A2 promoter. The concentration of heparin which would prevent multiple initiation events at the A2 promoter was determined by measuring the level of transcription from the promoter at different heparin concentrations. Figure 8 shows the results of an experiment in which enzyme/promoter complexes were formed in the presence of ATP and GTP for ten

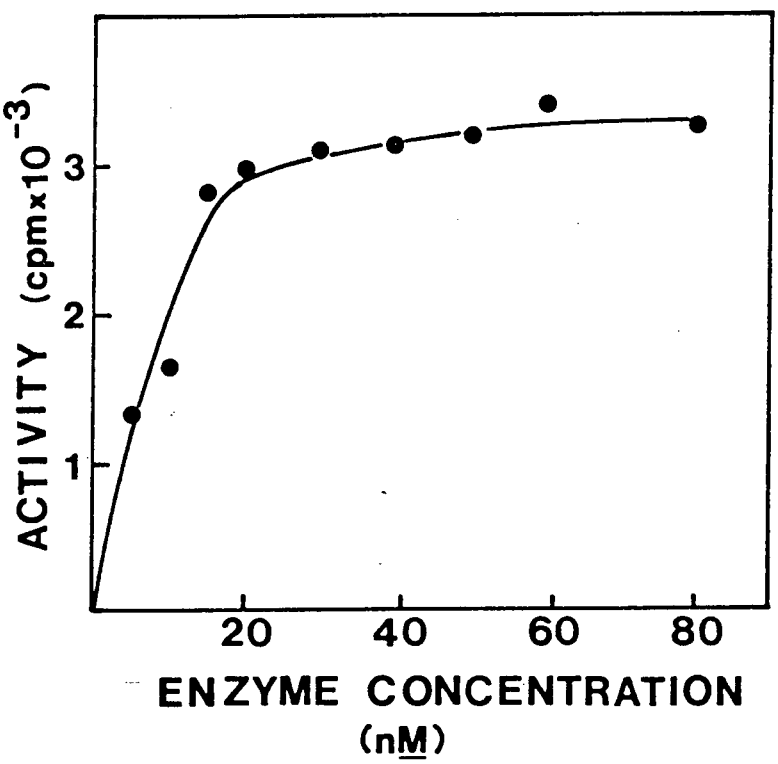
Figure 8. Effect of heparin concentration on transcription from the A2 promoter. Holoenzyme (core/sigma/delta) was incubated with the A2 promoter template, ATP and GTP for 10 minutes. Then heparin (at the indicated concentrations), CTP and UTP were added to the reactions to allow synthesis of run-off transcripts from the complexes formed during the initiation interval. Transcripts were separated on denaturing gels and the radioactivity in the A2-specific RNA quantitated and converted to the number of transcripts synthesized per A2 promoter, as described in Materials and Methods. The enzyme concentration was 50 n<u>M</u> and the DNA concentration 1.7 n<u>M</u>.


TRANSCRIPTS/PROMOTER
2.5
2.0
1.5
1.0
0.5
0
0
2
4
6
8
10
HEPARIN CONCENTRATION ug/ml

minutes. Initiation was terminated by the addition of UTP, CTP and heparin (at the concentrations indicated in the figure) to the reaction and elongation of initiated transcripts was carried out for 10 minutes. The number of transcripts synthesized per A2 promoter was determined as a function of the heparin concentration in the elongation reaction. In the absence of heparin, at 50 nM enzyme, approximately 2.4 transcripts/promoter were synthesized in a standard transcription reaction. As the heparin concentration was increased from 0 to 1 ug/ml the number of transcripts decreased by approximately 65% and then leveled off. In the experiment shown in Figure 8 the plateau occurred at approximately 0.8 transcripts per promoter. Based on the above data the heparin concentration chosen for subsequent experiments was 5 ug/ml, 10 to 20 fold lower than concentrations routinely used in studies of E. coli promoters (Miller and Burgess, 1978; Stefano and Gralla, 1980; Roe et al., 1985).

The effect of enzyme concentration on transcription from the A2 promoter. The amount of heparin to be used in the kinetic assays was determined at an enzyme concentration of 50 nM. Single round transcription assays were subsequently carried out at different enzyme inputs to show that 5 ug heparin/ml was sufficient to prevent multiple rounds of transcription at higher concentrations of enzyme. In the experiment shown in Figure 9 the reactions contained from 5 to 80 nM enzyme. The DNA was incubated with the

Figure 9. The effect of enzyme concentration on the level of transcription from the A2 promoter. Core/sigma/delta (at the indicated concentrations) was incubated with EcoRI-treated p328-5 DNA (1.7nM), ATP and GTP for 10 minutes to allow the formation of initiation complexes. Heparin, (5 ug/ml), CTP and UTP were then added to allow elongation from initiated complexes. The level of complex formation (activity) is expressed as the incorporation of [^{32}P]UTP into A2 transcripts.


ACTIVITY (cpmx10^{-3})
1
2
3
20
40
60
80
ENZYME CONCENTRATION
(nM)

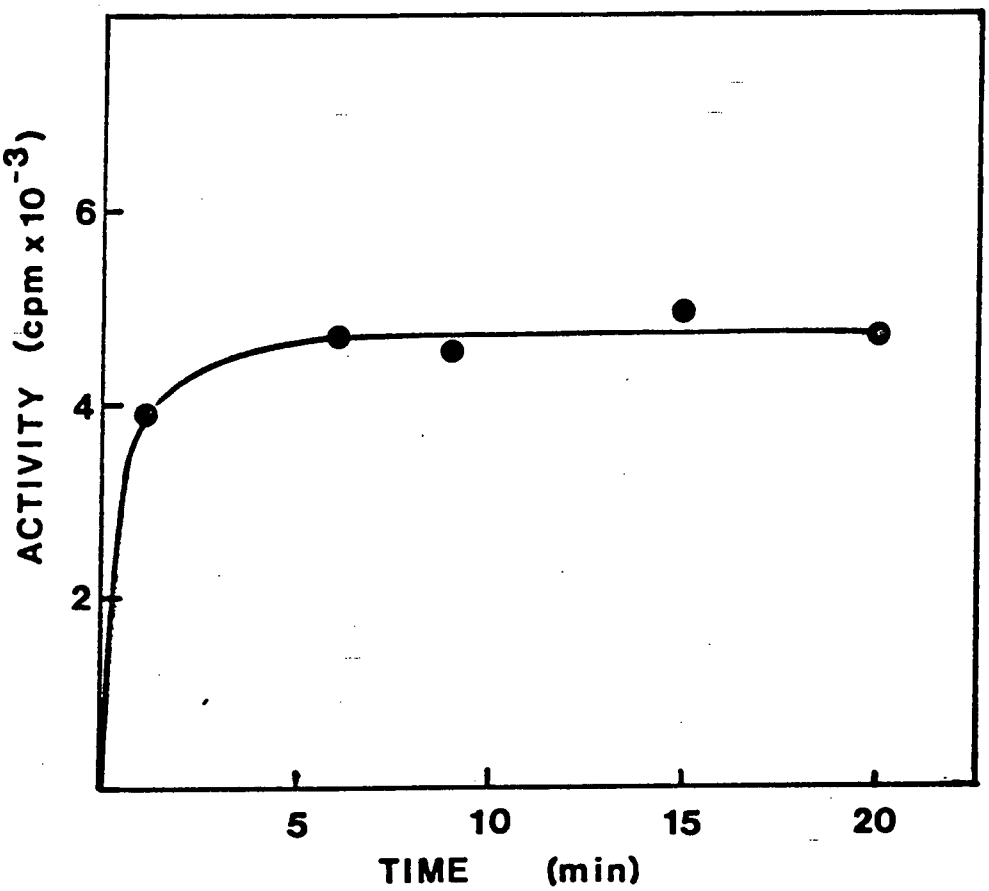
enzyme, ATP and GTP for 10 minutes before the CTP, UTP and heparin (5 ug/ml) were added to the reactions. As the enzyme concentration was increased from 5 to 30 nM the amount of transcription steadily increased. At enzyme concentrations greater than 30 nM the level of transcription approached a maximum value. In this particular experiment the maximum level of transcription, obtained at an enzyme concentration of 80 nM, was 0.7 transcripts/promoter

Initiation and Elongation of Transcription from the A2 Promoter. In order to carry out the kinetic analyses in this work it was necessary to determine the time intervals which would be sufficient to allow 1) maximal formation of initiated complexes and 2) completion of run-off transcripts.

The endpoint of the initiation reaction for the single round transcription assay was determined by incubating the A2 promoter template with the RNA polymerase, ATP and GTP for varying lengths of time (1 to 20 minutes), then adding heparin, CTP and UTP to allow elongation from the complexes which had formed during the initiation reaction. The elongation reaction was continued for 10 minutes. At an enzyme concentration of 10 nM the initiation reaction was not rapid but was essentially complete after five minutes (Figure 10). Therefore a 10 minute initiation time was chosen as a convenient endpoint for the initiation reaction.

The time course of the elongation reaction was also investigated. Initiation complexes were formed for 10

Figure 10. Effect of the initiation time interval on the level of transcription from the A2 promoter. Core/sigma/delta (10 nM) was incubated with EcoRI-treated p328-5 DNA (1.7 nM), ATP and GTP for various lengths of time before heparin, CTP and UTP were added to the reaction. The elongation reaction was carried out for 10 minutes. The ordinate represents the incorporation of [^{32}P]UTP into A2 transcripts (activity).


ACTIVITY (cpm x 10^{-3})
6
4
2
5
10
15
20
TIME (min)

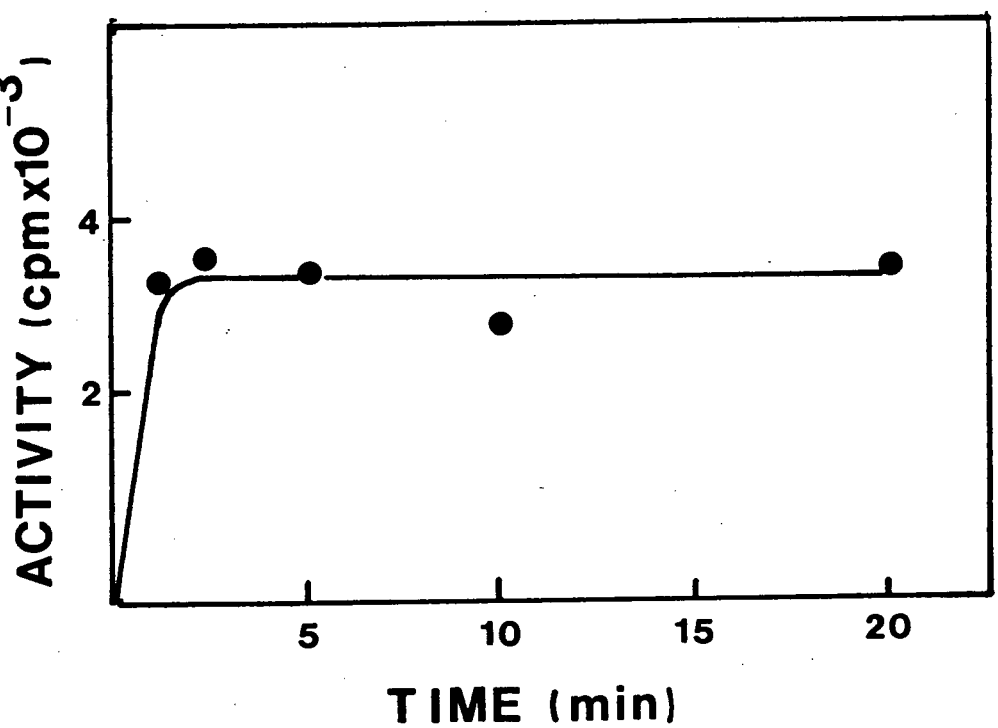
minutes prior to the addition of heparin, CTP and UTP. The time allowed for the subsequent elongation reaction was varied between 1 and 20 minutes. Figure 11 shows that the elongation reaction was very rapid. Transcription was complete within 1 minute and since the number of transcripts synthesized did not change as the incubation time was increased the elongation reaction was carried out for 10 minutes in all of the experiments subsequently described.

CHARACTERIZATION OF THE INITIATION REACTION AT THE A2 AND G2 PROMOTERS

A. Initiation at the A2 Promoter

Nucleotide requirements for stable complex formation at A2. The observation that heparin-resistant complexes were not formed when RNA polymerase was incubated with the A2 promoter suggested that the formation of stable (heparin-resistant) complexes at A2 might require the presence of the initiating ribonucleotide(s). The data also suggested that the transition to a heparin-resistant initiation complex might be slow relative to the rate at which heparin can inactivate the enzyme. The kinetics and extent of complex formation in the absence of nucleotides were therefore compared to complex formation in the presence of 1) ATP, 2) ATP and GTP or 3) ATP, GTP and CTP. The rate of complex formation was followed with a productive transcription rate assay (described in Materials and Methods) in which the

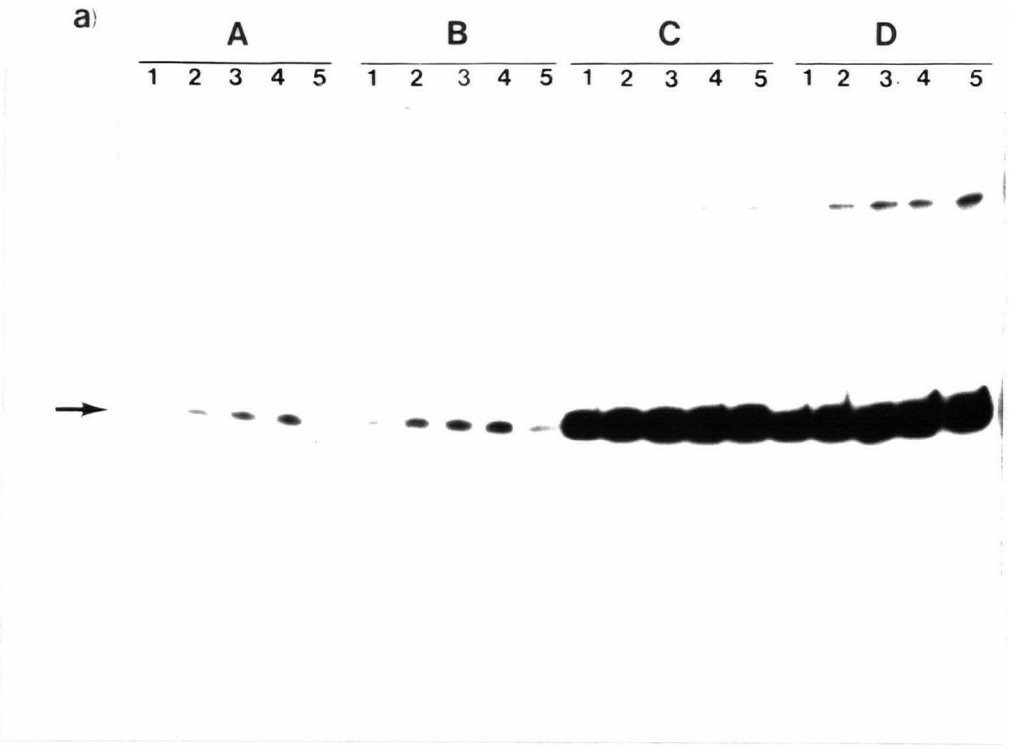
Figure 11. The effect of the elongation time interval on the level of transcription from the A2 promoter. RNA polymerase and the A2 promoter template were incubated for 10 minutes with ATP and GTP to form initiation complexes, prior to the addition of heparin, CTP and UTP. The elongation reaction was then carried out for various lengths of time (indicated on the figure). The incorporation of [α^{32}P]UTP into A2 transcripts was plotted as a function of the length of time allowed for elongation. The promoter concentration was 1.7 n<u>M</u> and the enzyme concentration 50 n<u>M</u>.

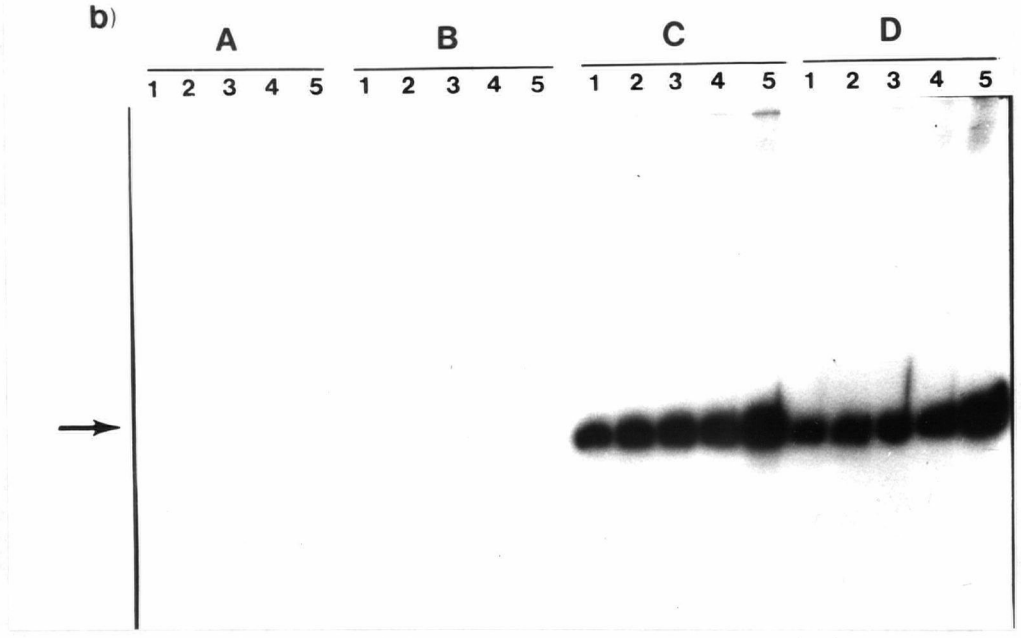

ACTIVITY (cpm x10^{-3})
4
2
5
10
15
20
TIME (min)

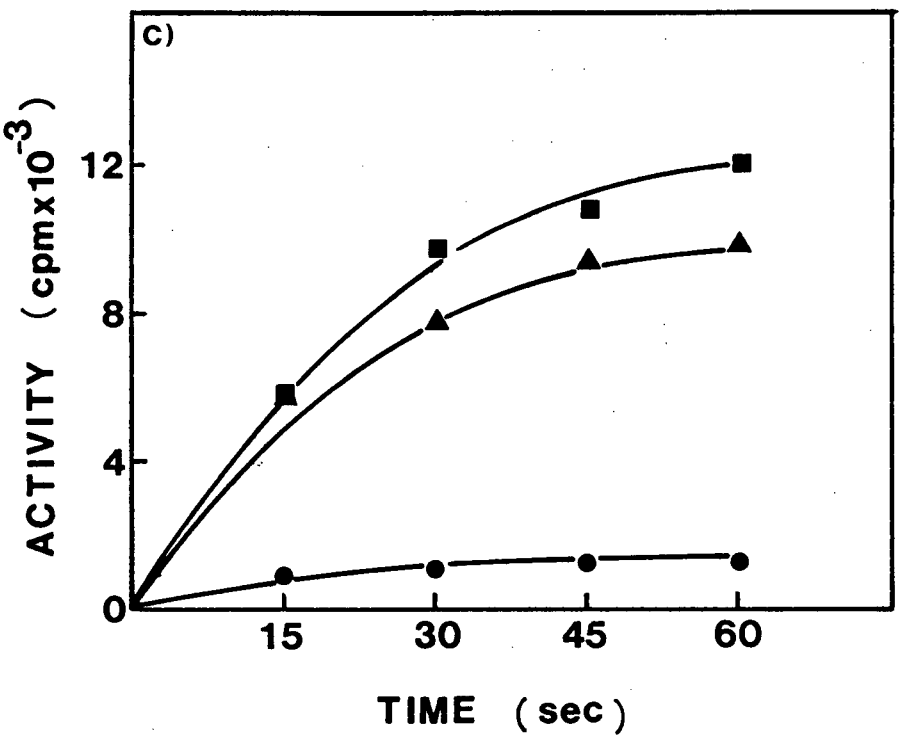
level of complex formation (as measured by the incorporation of [α^{32}P]UTP into run-off transcripts) was quantitated as a function of the time between the addition of the polymerase to the reaction and the addition of heparin and remaining nucleotides. Figure 12 shows the autoradiograms of the gels of products synthesized from complexes formed by (a) core/sigma or (b) core/sigma+delta in the presence of the combinations of nucleotides listed above. The complex formation reactions were carried out for the times indicated in the Figure legend. When complex formation was carried out in the absence of nucleotides or in the presence of ATP the A2 transcript (indicated by an arrow) was synthesized at very low levels, implying that under those conditions the RNA polymerase remains in a state which is sensitive to inactivation by heparin.

Incubating the enzyme with ATP and the template for 10 minutes (Figures 12a and b, lane B5) did not yield any more transcripts than when the enzyme and template were incubated together. This result showed that ternary complexes, composed of the enzyme, A2 promoter and ATP, were not heparin-resistant. It is assumed here that in the presence of ATP the enzyme is able to proceed through the initiation pathway to form ternary complexes at the A2 promoter. Complexes formed in the presence of ATP and the promoter will be subsequently designated A-initiated complexes. Similarly, complexes which are formed in the presence of ATP and GTP will be called AG-initiated complexes.

Figure 12. 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. Core/sigma (9n<u>M</u>) was incubated with DNA (1.7 n<u>M</u>) and the following ribonucleoside triphosphates: A) no nucleotides, B) ATP, C) ATP and GTP, D) ATP, CTP and GTP, and initiation 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 were separated on denaturing gels according to Materials and Methods. (b) Complex formation with core/sigma+delta (9n<u>M</u>) and 1.7 n<u>M</u> p328-5. Lanes are the same as in (a). (c) Plot of data from initiation time course (15 to 60 second time points) shown in (a). (●) complex formation in the absence of nucleotides or presence of ATP. (▲) complex formation with ATP and GTP. (■) complex formation with ATP, GTP and CTP. The ordinate represents the amount of [α^{32}P]UTP incorporated into the A2 transcripts.


a)
A
B
C
D
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5


b)
A
B
C
D
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5


C)
ACTIVITY (cpmx10^{-3})
12
8
4
0
15
30
45
60
TIME (sec)

The transcripts shown in Figure 12a were cut from the gel and quantitated as described in Materials and Methods. The amount of radioactivity incorporated into the transcripts was plotted against the amount of time allowed for complex formation (Figure 12c). These data showed that the number of heparin-resistant non-initiated complexes and A-initiated complexes formed during a 60 second reaction was very low (equivalent to 0.05 transcripts per promoter in this experiment).

The addition of GTP to the initiation reaction resulted in a 10-fold increase in the maximum yield of heparin-resistant complexes (see Figure 12c), suggesting that the AG-initiated complexes were much more resistant to heparin inactivation than were complexes formed in the absence of nucleotides or in the presence of ATP. The implication of these results is that the extent to which non-initiated or A-initiated complexes are converted to heparin-resistant, AG-initiated complexes after heparin is added to the reaction is not significant, although it is probably responsible for the low level of transcription observed from the non-initiated or A-initiated complexes.

The addition of CTP to the initiation reaction resulted in a further increase in activity over the activity observed in the presence of ATP and GTP. The increase was consistently observed with different experiments, although the extent of the increase varied between approximately 10 and 20%. It is not clear if complexes formed in the

presence of ATP, GTP and CTP are more resistant to heparin than are AG-initiated complexes or whether more of the complexes are formed.

In the presence of ATP, CTP and GTP the RNA polymerase is able to synthesize a 14 base transcript from the A2 promoter (Dobinson and Spiegelman, 1985). Hansen and McClure (1980) have shown, using a polyd(A-T) template, that sigma dissociates from the E. coli RNA polymerase after approximately 8 or 9 nucleotides have been incorporated into the RNA. It has been further hypothesized by von Hippel et al. (1982) that at this point the enzyme moves, essentially irreversibly, into an elongation state. It is possible that by the time a 14 base transcript has been synthesized from the A2 promoter the enzyme would have moved through initiation into an elongation phase. CTP was therefore omitted from subsequent initiation reactions to avoid introducing any rate-limiting steps which might be involved in the transition from an initiation to an elongation state.

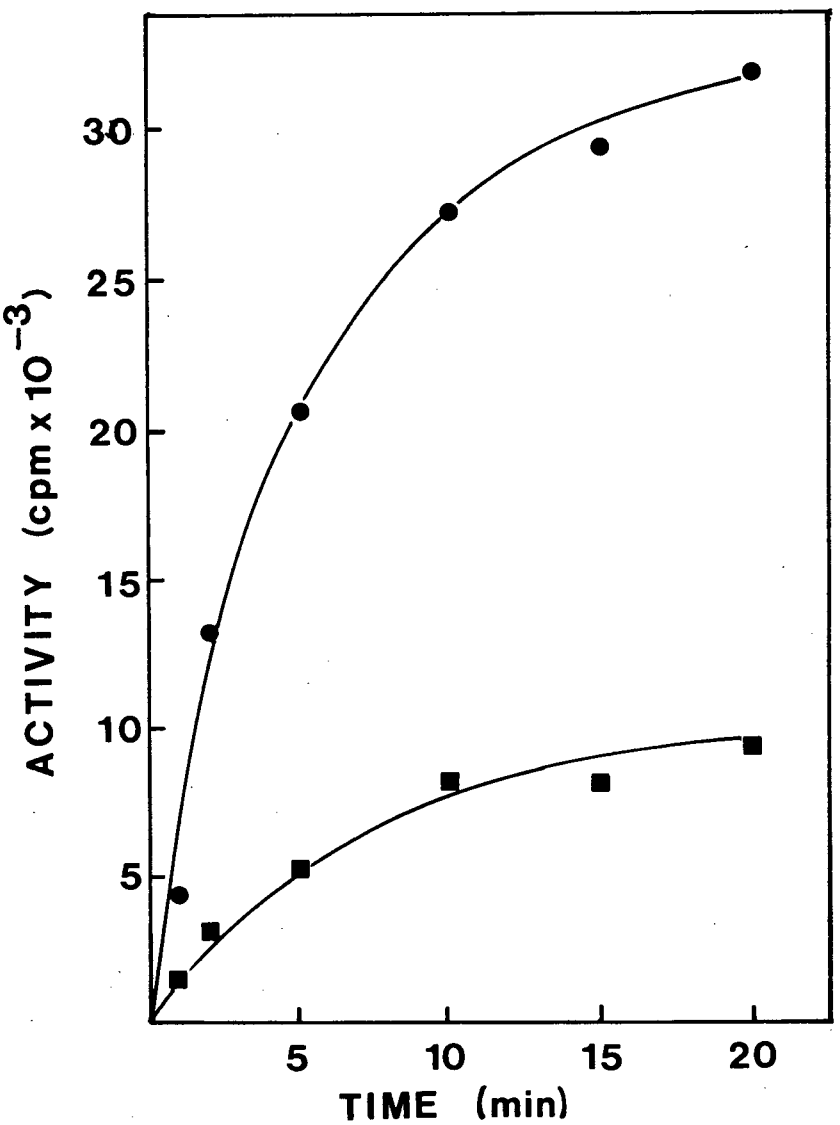
The Effect of Delta on Initiation at the A2 Promoter. The productive transcription rate assay was used to investigate the effect of delta on complex formation at A2. To ensure that any differences in the transcription kinetics could not be attributed to variability between different enzyme preparations, transcription by enzyme which had been depleted of delta (core/sigma) by DNA-cellulose or DEAE-sephadex chromatography, as described in Materials and

Methods, was compared with transcription from core/sigma which had been reconstituted with purified delta.

Standard, multiple round transcriptions were carried out, as described in Materials and Methods, with core/sigma and core/sigma+delta for varying lengths of time to determine if the addition of delta affected transcription from the A2 promoter. The amount of A2 transcript produced in the presence of delta was considerably lower than in the absence of delta and the rate at which transcripts were made was also slower (see Figure 13), indicating that at this particular promoter delta inhibited some step in the transcription pathway.

Although transcription was depressed in the presence of delta it was not possible to determine from the experiment described above which step(s) in the transcription pathway were being inhibited. Delta has been shown to inhibit the formation of stable non-specific enzyme/DNA complexes (Dickel, et al., 1980; Achberger and Whiteley, 1981) and it was possible that transcription from A2 was being inhibited in the same way. The effect of delta on complex formation at A2 was therefore investigated to determine if delta was inhibiting transcription by inhibiting the formation of initiation complexes. Complex formation was measured as a function of the time allowed for initiation, prior to the addition of heparin to the reaction. In this particular experiment (see Figure 14) approximately 0.8 transcripts per promoter were synthesized by core/sigma following a 60

Figure 13. Effect of delta on transcription from the A2 promoter. Standard, multiple round transcription reactions were carried out with core/sigma (●) and core/sigma+delta (■) as described in Materials and Methods, except that the times allowed for the reactions was varied between 1 and 20 minutes. Delta was added to core/sigma at a ratio of 0.4 mol delta:1 mol enzyme. The incorporation of [α^{32}P]UTP into the A2 transcripts is expressed as activity.

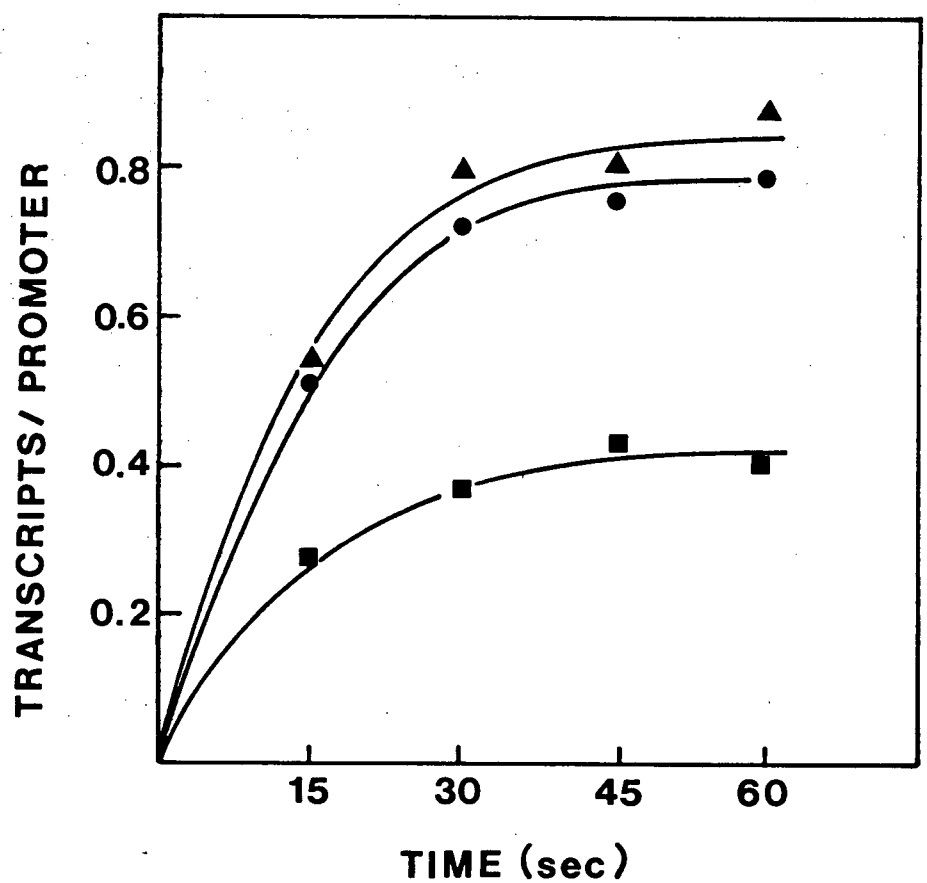

ACTIVITY (cpm x 10^{-3})
30
25
20
15
10
5
5
10
15
20
TIME (min)

second initiation. When core/sigma was reconstituted with delta the level of complexes formed during a 1 minute initiation dropped by 50% to approximately 0.4 transcripts/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 (with the heparin, CTP and UTP) delta had no effect on the level of transcription. To inhibit transcription from A2 delta must therefore be present at some point during the formation of initiation complexes, although the precise step in the initiation pathway which was affected could not be determined from the assay described above.

Kinetic studies of promoter-specific interactions by the E. coli RNA polymerase have led to the proposal that the formation of some polymerase/promoter open complexes proceeds through an unstable intermediate and that the formation of open complexes at such promoter sites exhibits pseudo-first order kinetics under conditions of enzyme excess (Stefano and Gralla, 1980; Hawley and McClure, 1980; McClure, 1980). Stefano and Gralla (1980) have further shown that the initial rate of open complex formation at these promoters can be measured by quantitating the amount of radioactivity which is incorporated into run-off transcripts after allowing complex formation for varying lengths of time (as described above). The fraction of unoccupied promoter sites ($[C_E-C_t]/C_E$) remaining after a

Figure 14. 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, delta added to the reaction with the heparin, CTP and UTP. The template DNA was 1.7 n<u>M</u> and the enzyme concentration 30 n<u>M</u>. Delta was added to core/sigma at a ratio of 0.8 mol delta: 1 mol core/sigma.


TRANSCRIPTS/ PROMOTER
0.8
0.6
0.4
0.2
15
30
45
60
TIME (sec)

binding time of t seconds can be calculated from the amount of radioactivity incorporated into the run-off transcripts synthesized from complexes formed during the binding reaction (C_t) and the maximum amount of radioactivity (C_E) incorporated into the RNA after an extended binding reaction. Since the loss of available promoter sites follows first order kinetics the time course of the binding reaction is linear when graphed on a semilogarithmic plot (Stefano and Gralla, 1982).

The data presented in Figure 15, taken from Figure 12, show that the formation of AG-initiated complexes at A2 followed pseudo-first order kinetics. The data also show that the addition of delta to core/sigma inhibited the rate at which initiation complexes formed at A2. The pseudo-first order rate constants (k_{obs}) for the initiation reactions could be calculated from the slopes of the lines (Stefano and Gralla, 1982) in Figure 15. K_{obs} is, intuitively, a measure of the velocity of the reaction. In this particular experiment 3.6 pmole delta was added per pmole core/sigma. K_{obs} was 0.019 s^{-1} for core/sigma and 0.006 s^{-1} for core/sigma+delta, indicating that as well as depressing the final number of complexes formed, delta depressed the rate of complex formation.

The effect of delta on the level of A2 transcript synthesized in a productive transcription assay was also measured at various enzyme concentrations. The results of this experiment are shown in Figure 16. In the absence of

Figure 15. Semilogarithmic plot of the data from the AG-initiation time courses shown in figures 12a and b. 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 minutes of initiation. The promoter concentration was 1.7 n<u>M</u> and the RNA polymerase was 9 n<u>M</u>. (●) core/sigma, (■) core/sigma+delta (3.6 mole delta/mole enzyme).

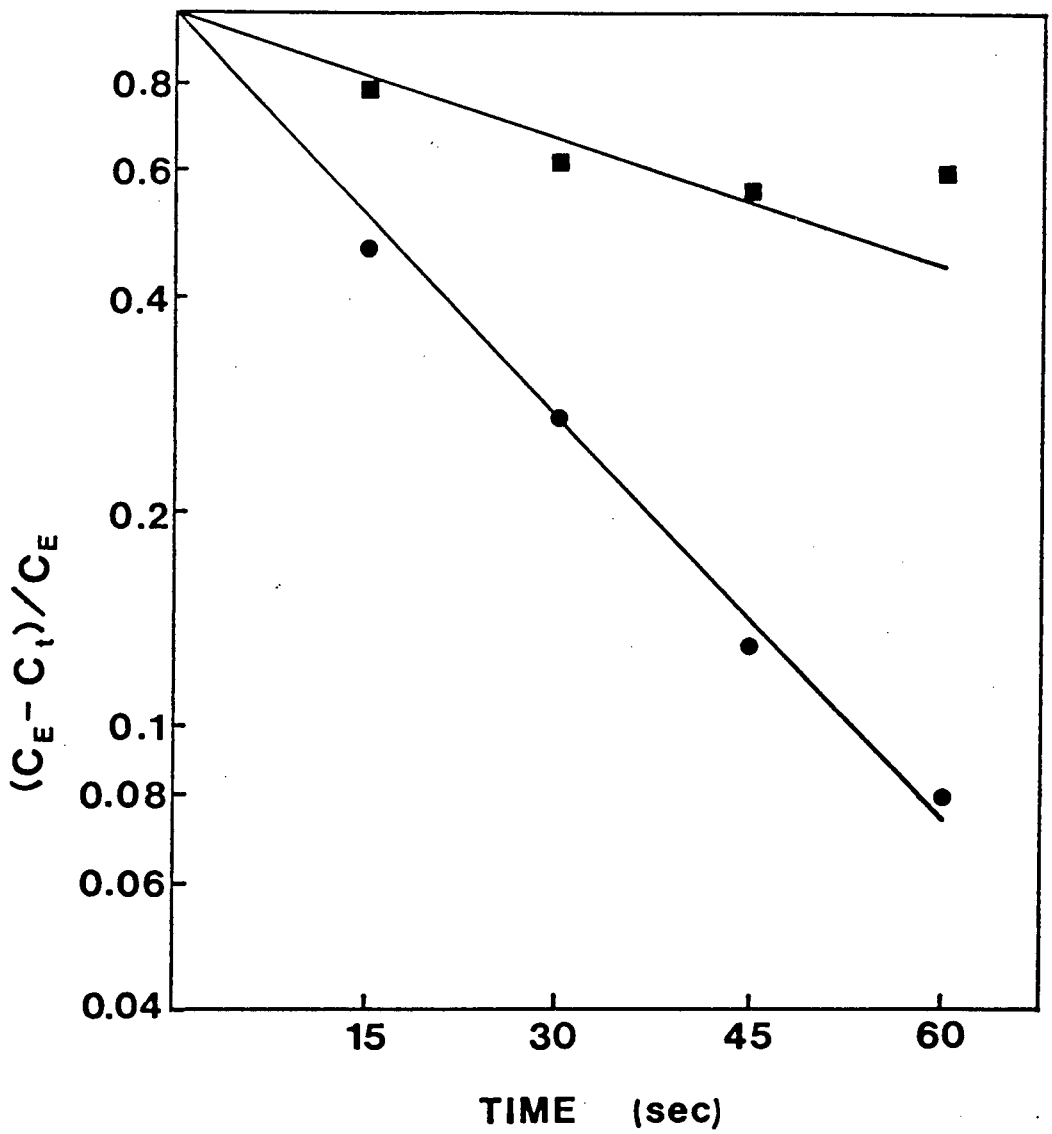
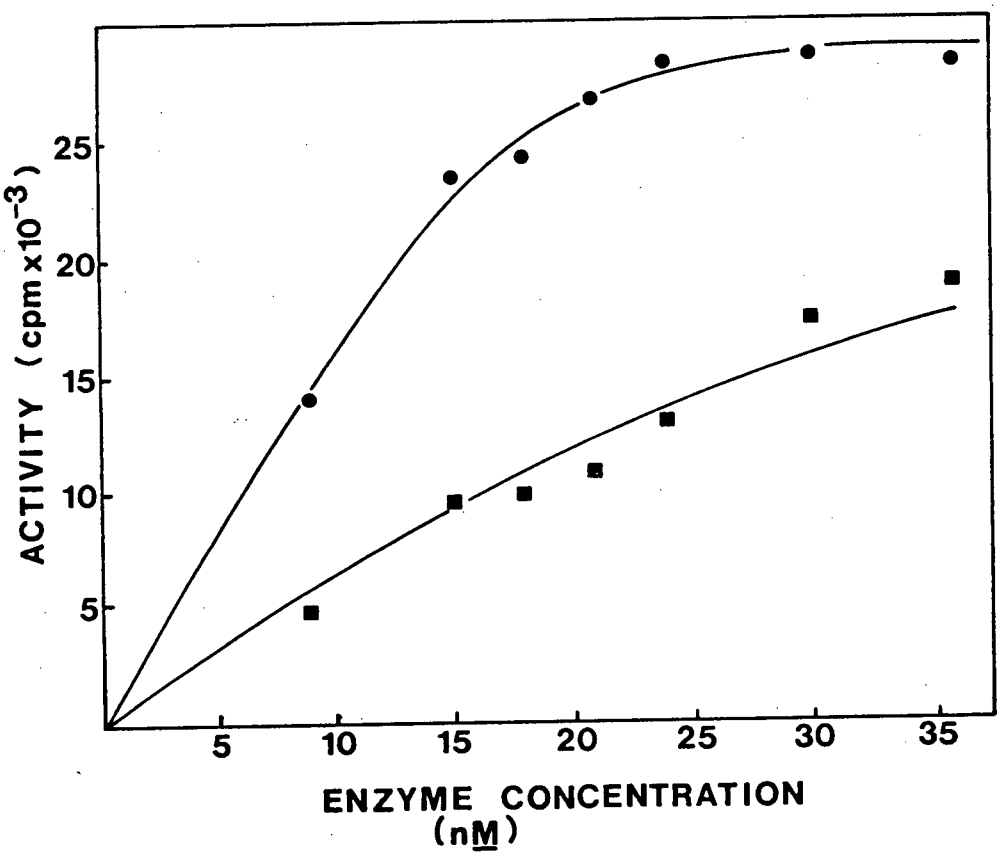

0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
$(C_E - C_t)/C_E$
15
30
45
60
TIME (sec)

Figure 16. Effect of delta on the maximum level of transcription from the A2 promoter at various enzyme concentrations. Core/sigma (at the indicated concentrations) was incubated in the presence (■) or absence of delta (●) under the conditions described previously for figure 9. Delta was added to core/sigma at a ratio of 0.5 mol. delta:1 mol. enzyme. The level of complex formation (activity) is expressed as the incorporation of [α^{32}P]UTP into A2 transcripts.

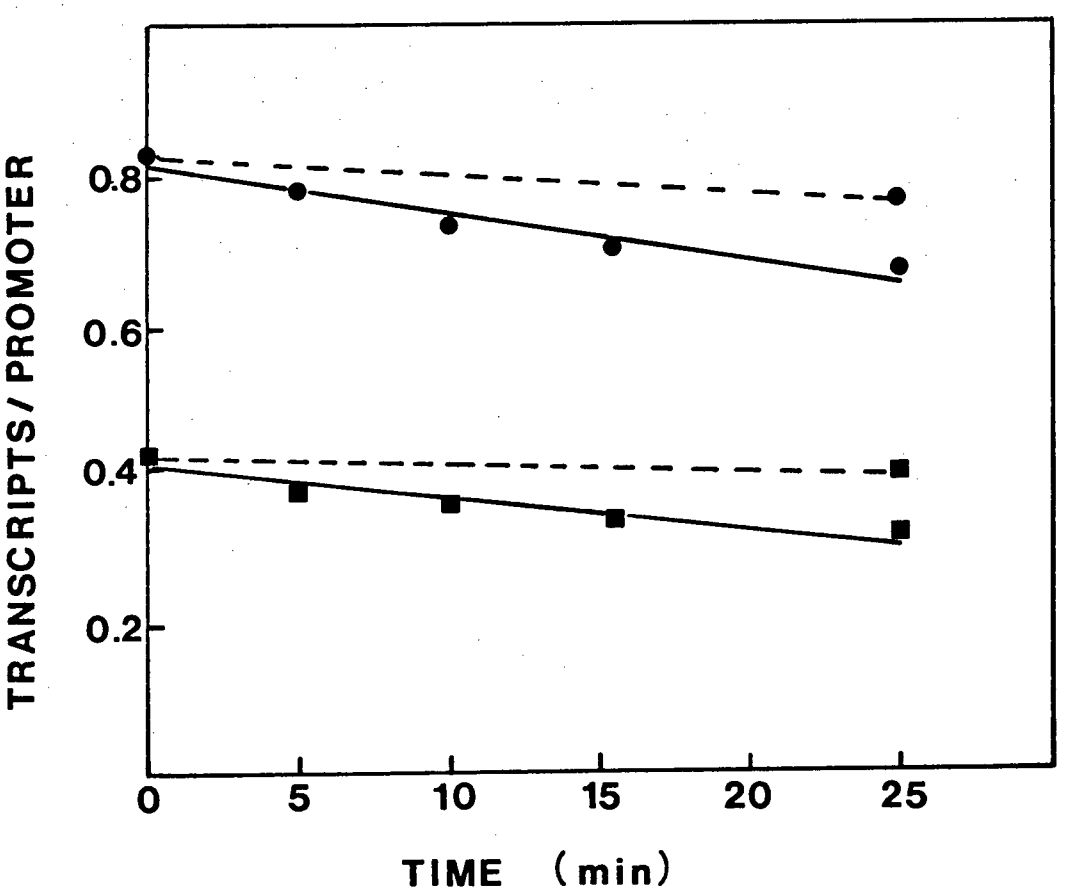

ACTIVITY (cpm x10^{-3})
25
20
15
10
5
5
10
15
20
25
30
35
ENZYME CONCENTRATION
(nM)

added delta the amount of transcription increased as the enzyme concentration increased, to a maximum of approximately 0.9 transcripts/promoter at an enzyme concentration of 36 nM. In the presence of added delta the level of transcription was lower at all of the enzyme concentrations tested and the maximum level of transcription obtained (at an enzyme concentration of 36 nM) was approximately 0.6 transcripts/promoter.

The Effect of Delta on the Stability of Initiated Complexes. One mechanism by which delta could inhibit complex formation would be by destabilizing the initiated complexes. The stability of the initiated complexes was therefore tested by incubating AG-initiated complexes in the presence of heparin for varying lengths of time before allowing the elongation reaction to occur. Enzyme which dissociated from the initiated complexes during the incubation would be inactivated by heparin and the loss of functional initiation complexes would be observed as a corresponding decrease in the number of transcripts per promoter.

An example of a time course of loss of initiated complexes in the presence of heparin is shown in Figure 17. When pre-formed initiation complexes were incubated in the presence of heparin the fraction of core/σ and core/$\sigma + \delta$ complexes decreased with increased incubation time. While delta reduced the level of complexes formed, the addition of delta to the enzyme did not appear to alter the stability of

Figure 17. Effect of delta on the stability of initiated complexes. Core/σ (●) or core/σ + δ (■) was incubated with EcoRI-treated p328-5 DNA (1.7 n<u>M</u>), ATP and GTP for 10 minutes to allow the formation of initiated complexes. Heparin was then added and at the times indicated samples were removed to allow elongation from initiated complexes which had not dissociated during the heparin treatment. Promoter concentration was 1.7 n<u>M</u> and the enzyme concentration was 20 n<u>M</u>. Delta was added to core/sigma at a ratio of 0.5 mol.:1 mol. enzyme. Complex formation was quantitated as the number of A2 transcripts synthesized per promoter. The solid line represents the loss of activity detected in the presence of heparin and the dashed line represents the loss of activity detected in the absence of heparin.


TRANSCRIPTS/ PROMOTER
0.8
0.6
0.4
0.2
0
5
10
15
20
25
TIME (min)

the complexes or their sensitivity to direct inactivation by heparin. Therefore delta must affect some other parameter in the initiation reaction. In the experiment presented in Figure 17 the core/sigma complexes seemed to be slightly less stable than core/sigma+delta complexes. However, this result was not consistently observed in repeat experiments and the difference was therefore not considered to be significant.

The Stability of Non-initiated Complexes at the A2 Promoter. Competition assays were carried out in an attempt to measure the stability of non-initiated complexes at the A2 promoter, with the intention of using the assay to determine if delta inhibited stable complex formation by increasing the dissociation rate of non-initiated complexes. The experiment was carried out as follows: core/sigma was first incubated with p328-5 DNA for five minutes. To ensure that there were sufficient promoter sites to bind all the available enzyme, the experiment was carried out under conditions of DNA excess, with the A2 promoter concentration at 8.5 nM and an active enzyme concentration of 5 nM. The competitor DNA (which also carried a promoter recognized by the sigma-43 RNA polymerase) was added to the A2 template and RNA polymerase, after the five minute binding reaction, to bind enzyme which dissociated from the A2 promoter. The concentration of the competitor template in the reaction was also 8.5 nM. At 15 second intervals after the competitor was added samples were removed and added to ATP and GTP to

allow enzyme which was bound to either of the promoters to form initiation complexes, thus trapping the enzyme at the promoters. The initiation reaction was carried out for ten minutes before heparin, CTP and UTP were added to allow elongation from the initiated complexes. The loss of A2 complexes during the competition reaction would be detected as a decrease in the number of A2 transcripts and a corresponding increase in the number of G2 transcripts.

The competitor DNA was a plasmid construct (obtained from G.B. Spiegelman, University of British Columbia) carrying the ϕ29 G2 promoter (Sogo et al., 1979; Davison et al., 1979, 1980; Garvey et al., 1985) cloned into pEMBL. This plasmid can be linearized downstream of the G2 promoter using the restriction enzyme HindIII. 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 results of one competition experiment are shown in Table II. When the enzyme (core/sigma) was incubated with the A2 template for five minutes prior to the 10 minute initiation reaction, 0.08 transcripts/enzyme were synthesized from A2. When the competitor and A2 templates were incubated together for five minutes the level of transcription from A2 dropped to 0.02 transcripts/enzyme whereas 0.2 transcripts were synthesized from G2. It should be noted that although the level of transcription from the A2 promoter was very low the observed decrease was

TABLE II

Template competition assay

Assay Conditions	Template	Pre-binding	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

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 (10 minute AG initiation followed by a 10 minute elongation reaction) with A2 alone or 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 minutes, then G2 was added and the binding reaction continued for an additional 15 seconds before allowing initiation to occur.

reproducible. The G2 template thus seemed to compete very effectively with the A2 promoter for the available enzyme when the two templates were added simultaneously to the reaction. The G2 promoter also acted as an efficient competitor when it was added to preformed A2/RNA polymerase complexes. Within 15 seconds 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. This result indicated that enzyme bound at the A2 promoters dissociated very rapidly. In fact, core/sigma dissociated from A2 so rapidly that a dissociation rate could not be ascertained. While it is possible that non-initiated complexes would be more unstable in the presence of delta, due to the limitations of the assay (it was not possible to sample at time intervals of less than 15 seconds) an increased dissociation rate would not be detectable. Although this particular assay was not sensitive enough to determine if delta affected complex formation by increasing the instability of the A2-polymerase binary complexes it did demonstrate the rapid equilibrium state of the non-initiated complexes at the A2 promoter.

The competition assay revealed two additional pieces of information about complex formation at the A2 and G2 promoters. When the RNA polymerase was not incubated with the A2 template prior to initiation, approximately 0.2 A2 transcripts were synthesized per enzyme (in the absence of

the G2 competitor). The enzyme activity thus decreased by 60% (to 0.08 transcripts/enzyme) when the template and enzyme were pre-incubated together for only five minutes, suggesting that the enzyme was inactivated very rapidly under conditions where heparin-resistant initiation complexes could not be formed. It was also observed that more transcripts were synthesized from the G2 promoter than from the A2 promoter (see Table II). This observation supports a previous report by Davison et al. (1980) that in vitro transcription from the G2 promoter was much more efficient than transcription from the A2 promoter at low enzyme concentrations.

Kinetics of RNA synthesis initiation at the A2 promoter (Tau plot analysis). As described above, a preliminary kinetic analysis presented in Figure 15 showed that the formation of initiation complexes at A2 followed pseudo-first order kinetics, analogous to open complex formation at the E. coli lacUV5 promoter.

Stefano and Gralla (1980) and McClure (1980) have proposed that the formation of open complexes at some E. coli promoters (such as lacUV5) proceeds as follows:

$$E + P \rightleftharpoons E\text{-}P_c \rightleftharpoons E\text{-}P_o$$

where $E\text{-}P_c$ is an unstable (closed) intermediate, $E\text{-}P_o$ is the open polymerase/promoter complex and the transition from the closed to the open complex is the rate-limiting step in the pathway.

Based on the above model Stefano and Gralla (1982) used a composite rate assay to investigate open complex formation at mutant lac promoters. This assay combines the productive transcription rate assay, in which the rate of complex formation is measured at different enzyme inputs, with the tau plot analysis of McClure (1980) to calculate the rate and equilibrium constants which characterize the formation of polymerase/promoter open complexes.

The Stefano and Gralla model now appears to be oversimplified with respect to the current model (McClure, 1985). Recent data support the hypothesis that the formation of open complexes proceeds through a minimum of three kinetically significant steps (Roe et al., 1984; Buc and McClure, 1985; Spassky et al, 1985). It should be noted however that the additional intermediate reported by those authors was only detectable at extremes of ionic strength and temperature. For the purposes of the kinetic analysis carried out by Stefano and Gralla (1982) the two-step model is therefore sufficient.

By analogy with the Stefano and Gralla model for open complex formation at mutant lac promoters, the initiation pathway, as measured by the AG-initiation assay, at the A2 promoter can be written as follows:

$$E+P \rightleftharpoons E\text{-}P_{*} \longrightarrow E\text{-}P_{i}$$

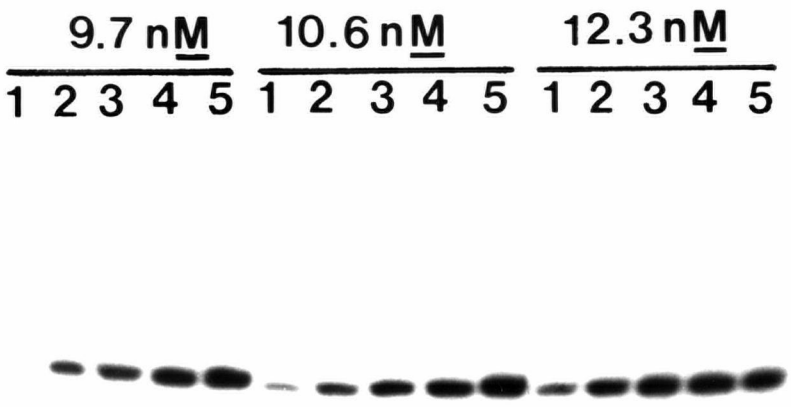
where $E\text{-}P_{i}$ is the AG-initiation complex. The intermediate ($E\text{-}P_{*}$) may not be comparable to the closed complex that is formed at the lacUV5 promoter (it could for example be an

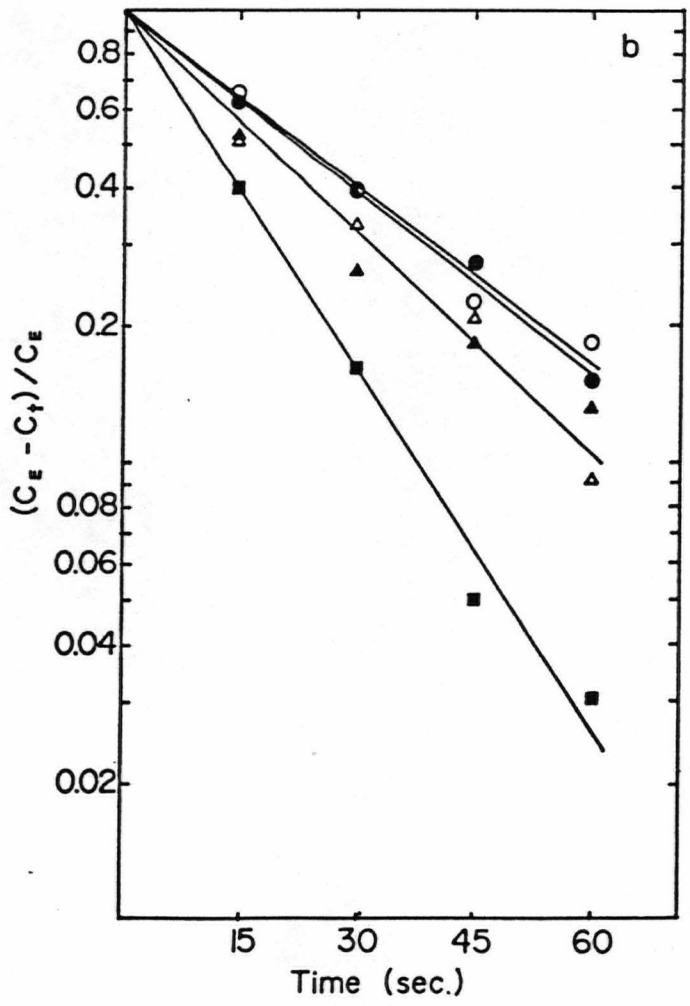
open complex in which the enzyme readily dissociates from the promoter). $E\text{-}P_{\#}$ is defined as the unstable intermediate whose conversion to $E\text{-}P_i$ is rate-limiting. The transition from the unstable intermediate to the initiated complex is assumed to be essentially irreversible and is written as a single step because the formation of other possible intermediates was not measured. It is unlikely that the binding of the initiating nucleotide and synthesis of the first phosphodiester bond is rate-limiting (McClure, 1980; Stefano and Gralla, 1980, 1982). The assumption therefore, is that although the endpoint of the AG-initiation reaction is an initiated complex rather than a non-initiated open complex, adding ATP and GTP to the reaction should not introduce additional steps which are potentially rate-limiting. It is also assumed that the AG-initiation assay reveals the same basic mechanism as the rate assay used in the work of Stefano and Gralla (1982). The initiation kinetics at the A2 promoter and the effect of delta on the kinetic parameters were therefore investigated using the tau plot analysis.

An example of the data for the measurement of the initial rate of initiation complex formation, at different polymerase inputs, is shown in Figure 18. The fraction of unoccupied promoter sites ($\{C_E\text{-}C_t\}/C_E$) was plotted on a logarithmic scale as a function of the time allowed for complex formation. The tau plot analysis was carried out by calculating the pseudo-first order rate constants (k_{obs})

Figure 18. (a) Autoradiogram of a polyacrylamide gel of the products from the assay for initiation complex formation, at three RNA polymerase concentrations. Enzyme was added to DNA, ATP and GTP and initiation allowed for 15 s (lane 1), 30 s (lane 2), 45 s (lane 3), 60 s (lane 4) or 10 min (lane 5). Heparin, CTP and UTP were added to allow synthesis of run-off transcripts from the complexes formed at the A2 promoter during the initiation interval. Transcripts were separated on gels as described in Materials and Methods. (b) Semilogarithmic plot of data from an initiation time course. The ordinate represents the fraction of available promoter sites ($[C_E-C_t]/C_E$) remaining after the period of initiation, calculated as in figure 15. The promoter concentration was 1.7 n<u>M</u>. RNA polymerase (active enzyme) concentrations were: 22.9 n<u>M</u> (■), 19.4 n<u>M</u> (△), 15.8 n<u>M</u> (▲), 10.6 n<u>M</u> (●), 9.7 n<u>M</u> (○).

a)


9.7 nM
10.6 nM
12.3 nM
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5


b
0.8
0.6
0.4
0.2
0.08
0.06
0.04
0.02
$(C_E - C_t)/C_E$
15
30
45
60
Time (sec.)

from the slopes of the lines obtained at each enzyme concentration, as described above. Tau values ($tau=1/k_{obs}$) were plotted against 1/enzyme concentration to generate plots such as the one shown in Figure 19 and the rate constants were derived as defined by Stefano and Gralla (1982). The y intercept on the tau plot is $1/k_2$ and the x intercept is $-1/K_{A*}$, where k_2 is the forward rate constant for the conversion of the intermediate to the stable complex ($E\text{-}P_* \quad E\text{-}P_i$), K_{A*} is the equilibrium dissociation constant for the intermediate (McClure, 1980; Stefano and Gralla, 1982) and the overall forward rate constant (K_{on}) for the reaction $E + P \longrightarrow E\text{-}P_i$ is k_2/K_{A*}.

Tau plots were generated for five different enzyme preparations. Table III shows the calculated k_2, K_{A*} and K_{on} values for each enzyme. The content of sigma in the various preparations was used to estimate the fraction of active enzyme in each preparation to facilitate comparison between the preparations. The K_{A*} values have therefore been normalized with respect to the sigma content of each enzyme. The delta content was also determined for four of the five enzymes, as described in Materials and Methods. Although the delta content of #4 was not determined, examination of the enzyme on an SDS-polyacrylamide gel stained with coomassie blue showed that the enzyme had approximately the same amount of delta as #5.

The data presented in Table III show that all preparations (with the exception of #3), had similar K_{A*}

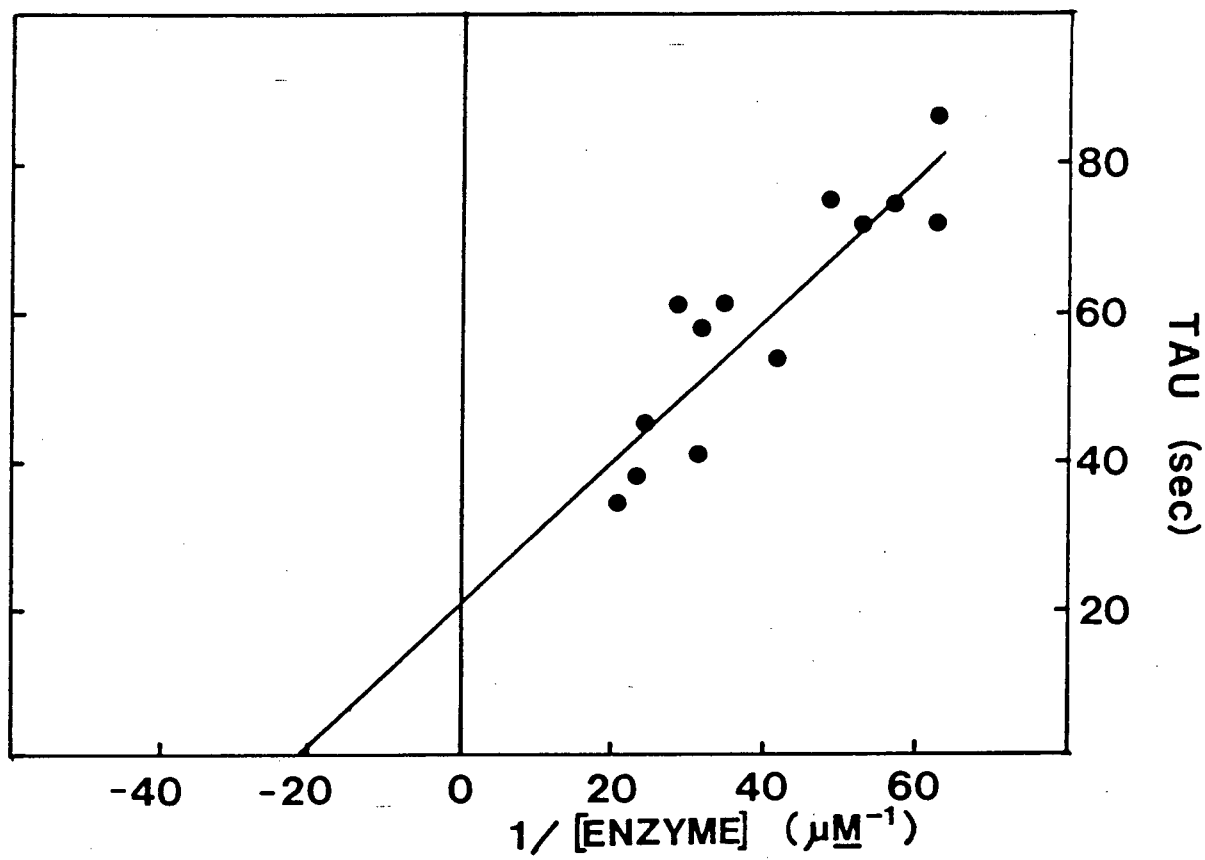


Figure 19. Tau plot analysis of initiation complex formation at the A2 promoter for one of the enzyme preparations (#1) shown in Table III. Each point represents a tau value, calculated from the initial rate of complex formation for the corresponding enzyme concentration, as determined by the run-off transcription assay. The line was calculated by linear regression.

TABLE III

Kinetic parameters observed for different preparations of Bacillus subtilis RNA polymerase

Enzyme	Sigma Content (%)	Delta Content (%)	k_2 (s^{-1})	K_{A*} (nM)	K_{on} ($x10^6$ $M^{-1}s^{-1}$)
1	88	66	0.049	47	1.0
2	48	57	0.083	21	4.0
3	48	60	0.035	6	5.8
4#	66	ND	0.238	55	4.3
5#	73	31	0.104	62	1.7

A tau plot analysis (described in text) was carried out for five different enzyme preparations. The kinetic paramaters k_2, K_{A*}, and K_{on} were calculated from the tau plots as described in text. Preparations indicated by the asterisks were depleted of delta as described in Materials and Methods. The delta content of #4 was not determined.

values whereas K_{on} ranged from 1 to 5.8 x 10^6 $M^{-1}s^{-1}$. The k_2 values ranged from .035 to .238 s^{-1}. All of these values are within the range of values calculated by Stefano and Gralla (1982) for several mutant lac promoters, suggesting that the modifications to the assay have not introduced any significantly slow kinetic steps. The content of delta did not seem to affect K_{on} or K_{A*}. The relationship between k_2 and delta content is less clear. The data presented in Table III suggest that the rate constant k_2 might increase as the content of delta decreases.

B. Characterization of the Initiation Reaction at the G2 Promoter

The G2 promoter has not been as extensively characterized as the A2 promoter in the in vitro transcription system, nor has the specific initiation site of the in vitro transcript been mapped. The size of the G2-specific transcript (approximately 120 bases) did suggest that transcription was being initiated accurately from the G2 promoter site which is located 130 basepairs upstream of the HindIII restriction site (G.B. Spiegelman, personal communication; Garvey et al, 1985). It was therefore decided to carry out an analysis of complex formation at the G2 promoter.

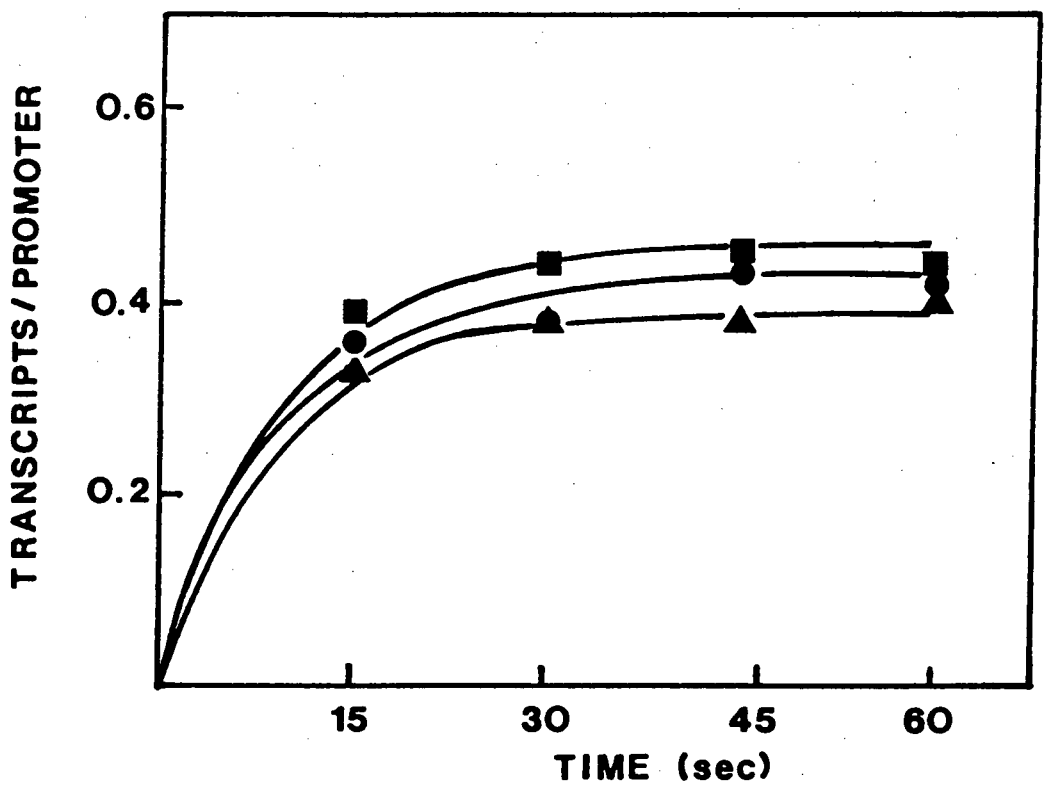
Nucleotide requirements for stable complex formation at the G2 promoter. Complex formation at G2 was analyzed with the same assay used to define the requirements for stable

complex formation at the A2 promoter. Complex formation in the absence of nucleotides was compared to complex formation in the presence of 1) GTP, the initiating rNTP (Davison et al., 1980; G.B. Spiegelman, personal communication) or 2) ATP and GTP. The level of complex formation (transcripts per promoter) was measured as a function of the time between the additions of the RNA polymerase and heparin and the remaining nucleotides to the reaction.

When holoenzyme was incubated with the G2 template (prior to the addition of heparin, ATP, CTP, GTP and UTP) the rate of complex formation and the final number of complexes observed were essentially the same as when GTP or ATP and GTP were included in the binding step, as shown in Figure 20. Unlike A2, the formation of heparin-resistant complexes at G2 apparently does not require initiation.

The Effect of Delta on Initiation at the G2 Promoter. The initial characterization of complex formation at the ϕ29 G2 promoter suggested that the kinetics of complex formation at the G2 and A2 promoters were different. The effect of delta on the formation of initiation complexes at G2 was therefore investigated with the composite rate assay, described above, which had been used to investigate complex formation at A2. 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 (G.B. Spiegelman, personal communication) the RNA polymerase is able to form a ternary complex at the G2

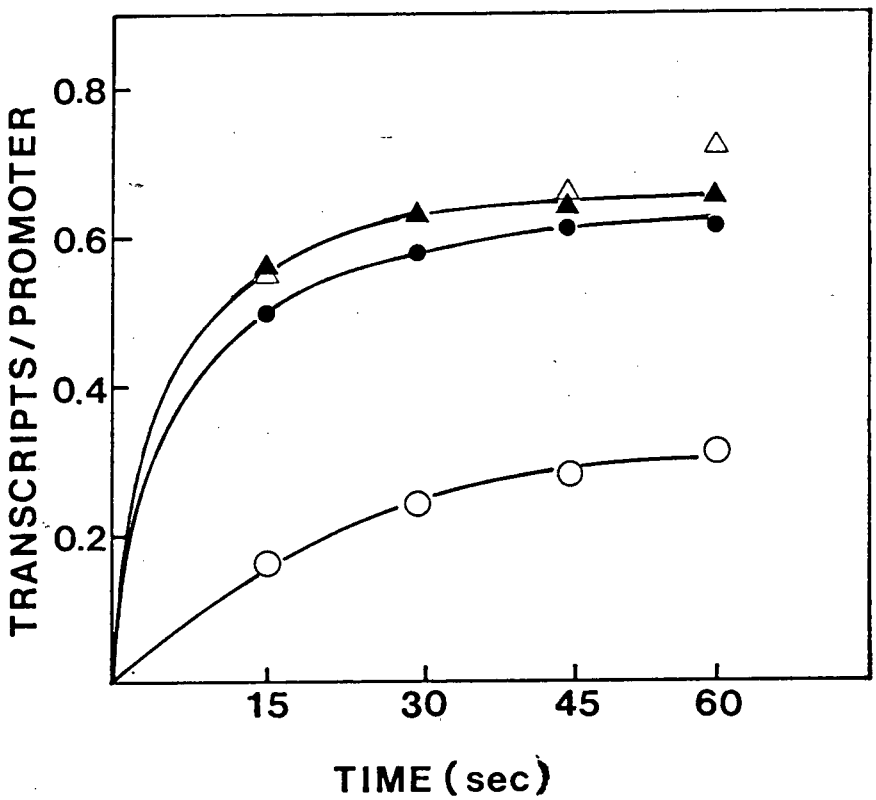
Figure 20. Nucleotide requirements for stable complex formation at the G2 promoter. The RNA polymerase (core/sigma/delta) was incubated with HindIII-treated G2 promoter template (1.7 n<u>M</u>) in the absence of nucleotides (●), in the presence of GTP (▲), or with ATP and GTP (■), for the times indicated. 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 n<u>M</u>.


TRANSCRIPTS / PROMOTER
0.6
0.4
0.2
15
30
45
60
TIME (sec)

promoter, similar to the 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 21. As a control the effect of delta was measured simultaneously with the A2 promoter. The number of transcripts per promoter was measured as a function of the time allowed for initiation, prior to the addition of heparin. Delta had no effect on complex formation at the G2 promoter, whereas the final level of complex formation at the A2 promoter was inhibited by approximately 50%.

Figure 21. Effect of delta on initiation from the 029 A2 and G2 promoters. Core/σ was incubated with (open symbols) or without (closed symbols) delta (at a ratio of 0.9 mol. delta: 1 mol. enzyme) in the presence of ATP, GTP and the A2 or G2 promoter for 15 to 60 seconds 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 n<u>M</u>. Enzyme concentration was 49 n<u>M</u>.


TRANSCRIPTS/PROMOTER
0.8
0.6
0.4
0.2
15
30
45
60
TIME (sec)

DISCUSSION

Methods for measuring initiation rates. The multi-step nature of the RNA synthetic pathway precludes a straightforward analysis of the transcription process. For example, in order to investigate the mechanism by which RNA polymerase forms specific complexes at promoters it is necessary to have an assay system which can quantitate the complex formation reaction independently of the elongation reaction. Three methods which have been devised for this purpose are the filter binding assay (Hinkle and Chamberlin, 1972a), the abortive initiation assay (Johnston and McClure, 1976: McClure et al., 1978) and the productive transcription rate assay (Stefano and Gralla, 1980).

The filter-binding technique is based on the observation that highly stable RNA polymerase/DNA complexes are retained on membrane filters whereas uncomplexed DNA does not bind to the filters. The formation of binary (non-initiated) or ternary (initiated) complexes at promoters can therefore be measured in the absence of elongation. This particular method has been used extensively for analysis of complex formation at E. coli phage T7 promoters (Hinkle and Chamberlin, 1972a,b) and at some B. subtilis phage promoters (for review see Doi, 1982).

Work by McClure and his colleagues (Johnson and McClure, 1976; McClure et al., 1978) has shown that at some E. coli promoters the RNA polymerase will reiteratively

synthesize and release di- or tri-nucleotides (abortive initiation products), without concomitant release of the enzyme. Abortive initiation is favoured in the presence of rifampicin or absence of ribonucleotides which would allow elongation of initiated transcripts, although abortive transcripts can be synthesized even when all four ribonucleotides are present (McClure and Cech, 1978; Carpousis and Gralla, 1980; Munson and Reznikoff, 1981). The abortive transcription products can be quantitated and used to measure the steady-state rate at which initiation occurs. This phenomenon has been exploited to study the rate-limiting steps in the transcription initiation pathway (McClure, 1980) and to identify promoter-containing DNA fragments (Cech et al., 1980).

While the two methods outlined above do allow the measurement of complex formation, they do not necessarily quantitate complexes which can produce complete transcripts, although it is assumed with the abortive initiation assay that the RNA polymerase would, under the appropriate conditions, be able to synthesize full-length transcripts. The productive transcription rate assay however, does measure functional complex formation because, as described above (see Results), only specific promoter/polymerase interactions which yield run-off transcripts are quantitated. This assay was therefore considered to be a simple and useful method which could be adapted for the

study of functional complex formation at Bacillus promoters.

Characterization of the in vitro transcription assay conditions. The ϕ29 A2 promoter which was cloned into pBR322 directs the synthesis of a discrete transcript, from EcoRI-treated p328-5, under the standard conditions described in Materials and Methods. This RNA has been shown to be 237 bases in length and to be initiated from the A2 promoter at the same site from which in vivo transcription is initiated (Dobinson and Spiegelman, 1985). Since the B. subtilis RNA polymerase is capable of initiating specific transcription from A2, the promoter was used to define the general conditions for the productive transcription rate assay and to study the effect of the delta peptide on specific polymerase/promoter interactions.

When heparin was added to preformed B. subtilis RNA polymerase/A2 promoter complexes very little transcription was detected (approximately 0.05 transcripts/promoter),even at heparin concentrations as low as 5 ug/ml (see Figure 12a and b, lanes A1 to A5). When complexes were formed in the presence of ATP and GTP and then added to UTP, CTP and increasing concentrations of heparin, transcription was maximally inhibited at a heparin concentration of 1 ug/ml. The level of transcription observed in the experiment shown in Figure 8 (0.8 transcripts per promoter) was similar to that observed by Stefano and Gralla (1980, 1982) and was maintained at heparin concentrations up to 20 ug/ml.

Because very little transcription was observed from complexes formed in the absence of nucleotides it appeared that free RNA polymerase and enzyme which was in a non-initiated state at A2 were both very sensitive to inactivation by heparin. The plateau region in the heparin concentration curve also suggested that the AG-initiated complexes are very stable and that RNA polymerase which is in such complexes at A2 is therefore insensitive to attack by heparin. Although the highest heparin concentration tested was 20 ug/ml it is possible that AG-initiated complexes at A2 are as insensitive to heparin as are open complexes at many E. coli promoters, which can be resistant to 100 ug/ml heparin (Stefano and Gralla, 1980, 1982).

The heparin concentration (5 ug/ml) for the subsequent assays was determined from an experiment in which the enzyme concentration was 50 nM (Figure 8). This concentration of heparin is sufficient to prevent multiple initiation events at enzyme concentrations up to 80 nM (see Figure 9).

The initiation time course described in Figure 10 was carried out at an enzyme concentration of 10 nM, the lowest concentration used in most experiments. The rate of initiation, as demonstrated by the tau plot analysis in Figures 18 and 19, is dependent on the enzyme concentration. It was therefore important for some experiments that the time interval chosen as the endpoint for the initiation reaction was sufficiently long to ensure that complex formation, even at low enzyme concentrations, had gone to

completion. The reaction was essentially complete by 5 minutes, as shown in Figure 10, therefore a 10 minute interval was considered to be a suitable endpoint.

The time course of the elongation reaction was also investigated for the following reason. The UTP concentration used in the assay was only 10 uM and although the elongation reaction does not require high nucleotide concentrations (Bremer, 1967) it has been shown that at low concentrations the RNA polymerase tends to pause at particular sites on the DNA template (Gilbert et al., 1974), slowing the overall elongation reaction. As seen in Figure 11, elongation of initiated transcripts was very rapid. The 10 minute elongation period used in the kinetic assays was therefore sufficient to allow all initiated transcripts to be completed.

Characteristics of heparin-resistant complex formation at the A2 promoter. Transcription from the A2 promoter, even in the absence of heparin, did not seem to be very efficient. In the experiment shown in Figure 8 only 2.4 transcripts per promoter were synthesized during a 10 minute standard reaction (an average rate of 1 base/second) when heparin was omitted from the reaction. This result was rather surprising since elongation from initiated transcripts seemed to be rapid (Figure 11). It would appear that either the formation of initiated complexes is slow or that a large fraction of enzyme does not synthesize complete transcripts. The observation that in the presence of

heparin 0.8 transcripts/promoter were synthesized does suggest that most of the initiated transcripts were completed, implying that the slow rate of transcription was due to a slow rate of initiation.

Stefano and Gralla (1979) observed that transcription from pre-formed complexes at the E. coli lacUV5 promoter during a single round transcription reaction proceeded slowly, with a $t_{1/2}$ of approximately one minute. They also observed that the synthesis of RNA from the pre-formed complexes was inhibited by rifampicin, which inactivates enzyme that has not initiated RNA synthesis (Sippel and Hartmann, 1968). Stefano and Gralla proposed that the rate-limiting step in the reaction must therefore be the formation of complexes which could initiate transcription. By analogy, the slow rate of transcription observed for the A2 promoter could be due to the slow formation of initiation complexes at the promoter.

The basic process leading to the initiation of RNA synthesis involves: 1) recognition of the promoter site by the RNA polymerase, 2) binding at the promoter site in a closed complex, 3) transition from the closed complex to an open complex in which the DNA helix at the promoter site is unwound and 4) transition to an initiation state, upon addition of ribonucleotides (for review see Chamberlin, 1976; von Hippel et al., 1982, McClure, 1985) The open complexes which are formed at many E. coli promoters are very stable; the release of RNA polymerase from these

complexes is slow, and the enzyme in the complexes is quite resistant to attack by heparin even in the absence of ribonucleotides (Miller and Burgess, 1978). However, these properties are not characteristic of all E. coli polymerase/promoter complexes. Kupper et al. (1976) have reported that the E. coli RNA polymerase does not form heparin-resistant binary complexes at a tyrosine tRNA promoter. Furthermore, some Bacillus subtilis RNA polymerase/promoter complexes can be destroyed by as little as 2.5 ug/ml heparin (Talkington and Pero, 1978). The initial observation that heparin inhibited transcription from ϕ29 A2 promoter/polymerase binary complexes therefore led to an investigation into the requirements for heparin-resistant complex formation at A2.

The preferred initiating nucleotide for the A2 promoter is ATP (Dobinson and Spiegelman, 1985). Incubating the enzyme with the A2 promoter template and ATP should therefore enable the enzyme to form a heparin-resistant ternary complex in which the DNA is unwound and the enzyme is associated with ATP and the promoter. As shown in Figure 12 complexes formed at the A2 promoter when the enzyme was incubated with ATP were as sensitive to heparin as were non-initiated complexes.

In the presence of ATP and GTP the RNA polymerase is able to synthesize the trimer pppAGA (Dobinson and Spiegelman, 1985) and under these conditions heparin-resistant complexes were formed (see Figure 12). Thus it

would appear that the formation of heparin-resistant complexes at A2 requires at least the formation of the first phosphodiester linkage and might require the synthesis of two phosphodiester bonds. This characteristic may be unusual but it is not unique; the formation of heparin-resistant complexes at the tyrosine tRNA promoter characterized by Kupper et al. (1976) also requires the synthesis of a dinucleotide. It has also been recently demonstrated that the formation of heparin-resistant complexes at the B. subtilis rrnB promoter requires nucleotides (V. Webb, personal communication).

There are three possible explanations for the observation that non-initiated complexes at the A2 promoter are not resistant to heparin. The first possibility is that the enzyme does not form open complexes at the promoter. The observation that A-initiated complexes are also sensitive to heparin would suggest, however, that the sensitivity of the non-initiated complexes is not simply a matter of whether the complex is closed or open.

The unusual sensitivity of the binary complexes to heparin could also occur if: 1) the enzyme forms open complexes which readily dissociate so that the enzyme is not protected from heparin attack or 2) the enzyme in the complexes is susceptible to direct attack by heparin and cannot rapidly initiate transcription in the presence of ribonucleotides. Pfeffer et al. (1977), for example, have shown that the E. coli RNA polymerase in open complexes at

the phage T7 A1 promoter is susceptible to direct attack by heparin. The low level of transcription from binary or A-initiated complexes at the A2 promoter would therefore be accounted for if the enzyme in open complexes was rapidly inactivated when heparin was added with the nucleotides which allow the polymerase to initiate transcription.

It was consistently observed that the number of complexes detected after a 10 minute reaction in which the enzyme and template were incubated in the absence of nucleotides or in the presence of ATP was lower in comparison with a 1 minute complex formation time (see Figure 12a and b). This result suggested that the enzyme was losing activity during incubation in the complex formation reaction. It has previously been reported (Hinkle and Chamberlin, 1972b, Stefano, 1981) that the E. coli RNA polymerase loses activity during in vitro assays, probably due to denaturation of the enzyme. The presence of DNA has been shown to protect the enzyme, although only if the enzyme can form stable complexes with the DNA, such as the heparin-resistant open complexes formed by the E. coli RNA polymerase at the lacUV5 promoter (Stefano, 1981). The observation that the B. subtilis enzyme lost activity when incubated with the A2 template is thus consistent with the hypothesis that the Bacillus subtilis RNA polymerase does not form stable complexes, as defined above, with the ϕ29 A2 promoter unless both ATP and GTP are included in the complex formation reaction.

The stability of the non-initiated complexes was investigated with a competition assay (see Table II). It was observed that the non-initiated complexes at the A2 promoter dissociated so rapidly that a dissociation rate could not be determined. These data further support the hypothesis that the non-initiated complexes formed at the promoter are unstable.

Characteristics of heparin-resistant complex formation at the G2 promoter. The competition assay (Table II) provided information regarding transcription from the ϕ29 G2 promoter. When the G2 template was added to pre-formed A2/polymerase complexes, transcription from A2 decreased from 0.08 to 0.02 transcripts/enzyme (see Table II). It was therefore expected that 0.06 G2 transcripts/enzyme would be synthesized if the decrease in the number of A2 transcripts reflected enzyme which had dissociated from A2 and subsequently bound to G2. However, 0.2 G2 transcripts were actually synthesized. Similarly, under conditions in which A2 was not pre-incubated with the RNA polymerase prior to initiation 0.2 A2 transcripts were synthesized, yet twice as many G2 transcripts were synthesized, under the same conditions, even when both templates were incubated together. These results suggested that the G2 promoter is considerably stronger than the A2 promoter. The RNA polymerase does not seem to efficiently recognize the A2 promoter, at least under conditions in which the enzyme:DNA ratio is low.

It has been hypothesized that delta would inhibit transcription from weak promoters, as well as from non-specific sites on the DNA, but have little effect on transcription from strong promoters (Achberger et al., 1982). The results of the competition assay indicated that the G2 promoter was stronger than the A2 promoter. It was therefore of interest to characterize initiation at the G2 promoter and to determine if, under the conditions of our assay system, delta would inhibit complex formation at G2.

The number of complexes observed when the RNA polymerase was incubated with the G2 template in the absence of nucleotides was essentially the same as when the enzyme and DNA were incubated with GTP or with GTP and ATP (Figure 20). Thus it appeared that, unlike the A2 promoter, the non-initiated complexes at the G2 promoter were as resistant to heparin as were initiated complexes. There is then, a distinct difference between the binary complexes which are formed at the A2 and G2 promoters. It could be speculated that the strength of the G2 promoter could be due, in part, to the ability of the B. subtilis RNA polymerase to associate with that promoter in binary complexes which are not readily dissociated and are therefore resistant to heparin.

The effect of the delta subunit on complex formation at the A2 and G2 promoters. Transcription from the A2 promoter was shown to be inhibited by delta in an in vitro multiple round transcription assay (see Figure 13). There is no

round transcription assay (see Figure 13). There is no trivial explanation for the inhibition since the delta preparation was shown to be free of DNase, RNase and proteolytic activity (see Table I and Figure 7). This experiment did not provide information as to which step in the transcription pathway was being inhibited, however there is considerable evidence that delta acts prior to initiation (Spiegelman et al., 1978; Achberger et al., 1982). It was therefore most likely that delta affected transcription from the A2 promoter by inhibiting formation of AG-initiated complexes at A2.

The data presented in Figure 12 showed that both core/sigma and core/sigma+delta required ATP and GTP to form heparin-resistant complexes at A2, suggesting that if delta affected the process of initiation it was in a quantitative, rather than qualitative manner. The data from the productive transcription rate assay shown in Figure 14 demonstrated that complex formation at A2 was inhibited by delta but not if it was added to the reaction after initiation complexes had been formed, indicating that delta directly affects some step in the initiation pathway.

An alternative explanation for the data in Figure 14 could be that enzyme which is associated with delta during initiation elongates RNA chains less efficiently, resulting in the synthesis of fewer complete transcripts by core/sigma+delta during the 10 minute elongation reaction. This explanation seems implausible for several reasons.

First, the A2 transcript is only 237 bases long and should easily be completed during the elongation interval. There was also no detectable increase in RNA products smaller than 237 bases (on polyacrylamide gels), although it is possible that such a change might not be noticeable if the enzyme paused or terminated transcription at random along the template. It has also been reported that core/sigma/delta synthesizes more large transcripts *in vitro* from phage SP82 DNA than does core/sigma (Spiegelman *et al.*, 1978). The latter data suggested that, rather than inhibiting elongation, delta either stimulated elongation directly or enhanced selection of strong promoter sites from which full-length transcripts could be synthesized. There is thus no prior evidence to support the hypothesis that delta might directly inhibit the elongation reaction.

Although the addition of delta to core/sigma clearly decreased the rate at which initiation complexes were formed at the A2 promoter (see Figure 15), it was not obvious which step(s) in the initiation pathway were altered. One possibility was that delta inhibited the initiation rate by destabilizing initiated or non-initiated complexes. As described in Results, the stability of the initiation complexes was tested directly (see Figure 17) and there was no evidence that delta altered the stability of those complexes.

Spiegelman and Whiteley (1979) studied the subunit composition of the *Bacillus subtilis* RNA polymerase during

transcription, using glycerol gradient sedimentation analysis, and observed that delta was released from the enzyme prior to initiation. It has also been shown that the non-initiated complexes formed by core/sigma/delta at an SP82 early gene promoter are significantly different from core/sigma complexes with respect to the number of sites at which the enzyme contacts the promoter. The initiated complexes formed by the core/sigma and core/sigma/delta enzymes are virtually the same (Achberger et al., 1982). These data suggest that delta acts prior to the formation of initiated complexes, and the observation that delta did not affect the stability of initiated complexes at the A2 promoter is consistent with that hypothesis.

The effect of delta on the overall level of transcription from the A2 promoter (see Figure 14 for example) was unexpected. The enzyme concentration curves in Figure 16 show that adding delta lowered the level of transcription and changed the point at which the enzyme saturated the promoter. The level of transcription from the A2 promoter has been shown to be affected by the enzyme concentration, although at high enzyme concentrations the level of transcription approaches a plateau value (see Figure 9). It was also observed throughout the course of this work that the maximum yield of A2 transcripts from a productive transcription assay only rarely attained the theoretical value of one transcript/promoter. In general, the level of transcription from holoenzyme, at high

concentrations of enzyme, ranged between 0.5 and 0.8 transcripts/promoter, whereas transcription from core/sigma was somewhat higher, usually from 0.7 to 1.0 transcript/promoter. It appeared therefore that delta might change the effective enzyme concentration in the reaction, that is, the enzyme that is capable of synthesizing RNA from the A2 promoter. This possibility is consistent with the data shown in Figures 15 and 18; adding delta to the reaction has the same effect as lowering the concentration of enzyme in the reaction; the rate of complex formation at the A2 promoter decreases.

Delta could inhibit both the extent and rate of complex formation in one of three ways: 1) core/sigma/delta could interact with the promoter but not initiate transcription, 2) enzyme which is associated with delta might be unable to interact with the promoter, or 3) delta could act by decreasing the rate of the reaction, thereby interfering with the formation of AG-initiated complexes.

The data from the tau plot analysis presented in Table III suggested that delta did not affect the rate of initiation complex formation at the A2 promoter by altering the equilibrium dissociation constant, K_{A*}, as there was no correlation between K_{A*} and delta content. It therefore appeared unlikely that core/sigma/delta interacted with the A2 promoter but was unable to initiate transcription or that core/sigma/delta was not able to interact with the promoter. Although either mechanism would explain the observation that

delta alters the rate of formation of AG-initiated complexes and the number of complexes formed at the A2 promoter, one would expect that if delta inhibited complex formation in such a manner, enzyme which had a high delta content would have a higher K_{A*} than enzyme which had been depleted of delta. The data presented in Table III are therefore not consistent with the above interpretation.

There appeared to be a correlation between the k_2 values and the delta content, although the data were not definitive. With the exception of enzyme preparation #3, which also had a low K_{A*} in comparison to the other enzymes, the k_2 value was lower for those enzymes which had approximately 60% delta than for the enzymes which had been depleted of delta. It is therefore possible that delta slows the rate of complex formation to such an extent that productive transcription is interfered with at the A2 promoters.

The interpretation that delta affects complex formation by altering the rate at which non-initiated complexes are converted to AG-initiated complexes must be made cautiously. The tau plot analysis has previously been used only for investigations into the interactions of the E. coli RNA polymerase at different promoters. It is quite possible that the sigma content or sigma/delta ratio, as well as the delta content of the different B. subtilis RNA polymerase preparations, would affect the kinetic parameters. As a

consequence, the tau plot analysis may be inadequate for comparisons between different enzyme preparations.

It should be noted that another potential problem with the tau plot analysis is that it is assumed that all of the active enzyme which is added to the reaction is available for interaction with the promoter of interest. In this assay non-specific transcription could therefore affect values for kinetic parameters which have concentration-dependent terms, specifically K_{A*} and K_{on}. The data presented in Figure 12a and b show that there is indeed some non-A2 RNA which migrates above the A2 transcript on polyacrylamide gels. The calculated values for K_{A*} and K_{on} may therefore be overestimated and underestimated, respectively.

Delta is obviously not a general inhibitor of promoter-specific transcription. Complex formation at the G2 promoter was shown, in contrast with complex formation at A2, to be unaffected by the presence of delta (see Figure 21). This result supports the hypothesis that delta would not inhibit complex formation at strong promoters (Achberger et al., 1982).

The chromosomal DNA of B. subtilis has a relatively high A+T content of approximately 57% (Geiduschek and Ito, 1982) and also contains single-stranded regions and nicks which could bind RNA polymerase non-specifically (Doi, 1982). Delta has been proposed to play a role in enhancing the specificity of the B. subtilis RNA polymerase by

preventing transcription, or at least the formation of stable complexes, at non-specific sites on the Bacillus chromosomal DNA (Achberger et al., 1982; Doi, 1982). The implication of this hypothesis is that non-specific interactions with nicked DNA or A-T rich sequences interfere in some way with promoter location. It would therefore be expected that enzyme which is associated with delta would transcribe promoters more efficiently, while non-specific transcription would be reduced.

The effect of delta on non-specific transcription was observed in the assay used in this work. Although most of the RNA which was synthesized originated from the A2 promoter there was, as discussed above, some non-specific transcription that probably originated from the plasmid vector. It was observed that when core/sigma was reconstituted with delta the overall level of transcription, both specific and non-specific, from the A2 promoter template decreased. Thus delta appears to inhibit non-specific interactions and A2 promoter-specific interactions equally well.

It was also observed that core/sigma+delta did not form initiation complexes any more rapidly at the G2 promoter than did core/sigma. This result would suggest that non-specific 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. This result does not, however, rule out the possibility that

alter the kinetics of promoter association if another template were used.

Williamson and Doi (1978) investigated the interactions between core, sigma and delta and were able to demonstrate that delta had a greater affinity for core than did sigma and could actually displace sigma from a core/sigma complex. They also observed that core/delta could bind to DNA with the concomitant release of delta. Based on these data it was proposed that delta directed the core enzyme to or near the promoter and that sigma bound to the complex, after delta was released, to allow initiation of RNA synthesis (Williamson and Doi, 1978). Some of the data presented here regarding the effect of delta on complex formation at the A2 promoter are consistent with that model. If delta competes with sigma for a binding site on core and core/delta binds to the A2 promoter less effectively than does core/sigma, then adding delta to core/sigma would inhibit the rate of complex formation at A2. The data obtained for complex formation at the G2 promoter, however, would suggest that such a mechanism would pertain only to certain promoters.

An alternative mechanism to explain the effect of delta could be that delta is not released efficiently from core/delta complexes at A2 but is rapidly released from G2 promoter complexes. The efficiency of delta release would therefore determine the rate at which sigma could bind to the core/promoter complexes to allow initiation of RNA synthesis. This particular mechanism would be consistent

synthesis. This particular mechanism would be consistent with the observed effect of delta on complex formation at both the A2 and G2 promoters. It is nevertheless difficult to reconcile the model with the observations that it is different sigma factors which confer promoter selectivity on the Bacillus RNA polymerase (Losick and Pero, 1981). The subunit composition of promoter-bound enzyme is therefore uncertain and will only be resolved by an investigation of the structure of the polymerase at the promoters.

Model for complex formation at the A2 and G2 promoters. Based on the data presented above and previous information about delta, the role of delta can be considered to include a modulating function to aid the RNA polymerase in discriminating between specific promoters, as has been postulated by Williamson and Doi (1978) and Achberger et al. (1982). A model for the effect of delta on complex formation at the A2 and G2 promoters is presented in Figure 22. For the sake of simplicity it is assumed that core/sigma and core/sigma/delta interact with the A2 and G2 promoters.

In the case of the G2 promoter the formation of initiation complexes can be interpreted to be analogous to the model presented by McClure (1985). 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. Delta is

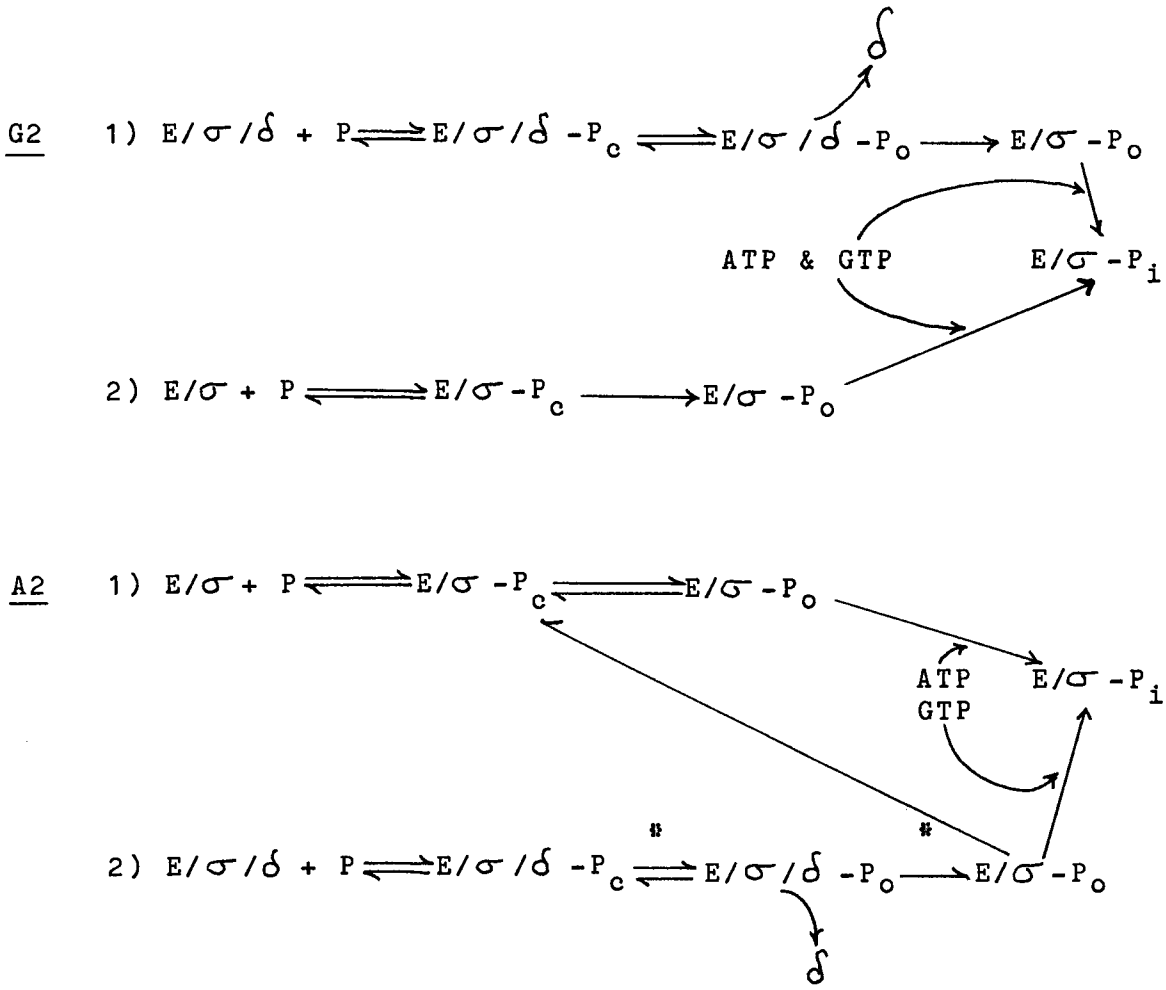


Figure 22. Model for the effect of delta on initiation at the A2 and G2 promoters. The promoters and core enzyme are designated P and E, respectively. P_c, P_o and P_i represent the closed, open and initiated complexes. The steps in the reactions which are hypothesized to be essentially irreversible are indicated ($\longrightarrow$) and the potential points at which delta could act to slow the reaction at the A2 promoter are indicated by an asterisk.

released from the complex at some point during this sequence of events and in the presence of the appropriate nucleotides the enzyme is able to initiate transcription.

It has been shown by Achberger et al. (1982) that open complexes formed by core/sigma/delta at an SP82 promoter are significantly different from core/sigma open complexes with respect to the sites at which the enzyme contacts the DNA. This result shows that at some point in the pathway enzyme that is associated with delta is bound to the promoter. An additional open complex intermediate has therefore been included in the pathway to account for those observations. It could further be postulated that the release of delta, which is associated with one of the transition steps, is necessary for initiation.

Enzyme which is not associated with delta would also bind efficiently to the "strong" G2 promoter and initiation would proceed by pathway 2) shown in Figure 22. Although it is not possible to define the precise sequence of events at the G2 promoter or how they might compare to the situation with E. coli it does appear that the basic process is not unlike complex formation at the lacUV5 promoter, for example.

The proposed sequence of events at the A2 promoter is also shown in Figure 22. In the case of core/sigma the enzyme interacts with the A2 promoter to form a closed complex which undergoes a transition to an open complex. The closed and open complexes are kinetically unstable but

the final open complex can form a stable (heparin-resistant) initiation complex in the presence of ATP and GTP.

Core/sigma/delta is able to bind to the A2 promoter but the rate at which core/sigma/delta complexes initiate transcription is considerably slower than the rate at which core/sigma forms initiation complexes. It is postulated that enzyme which is associated with delta forms complexes with the promoter that are not easily converted to open complexes that can initiate transcription.

There are two possible mechanisms by which delta could alter the rate of the reaction. In the first case the unstable intermediate in the productive transcription rate assay, analogous to that proposed by Stefano and Gralla (1980, 1982), is postulated to be a closed complex. The transition from that complex to an unstable open complex would therefore be the rate-limiting step in the reaction. The kinetic parameters K_{A*} and k_2, for core/sigma/delta would thus refer to the core/σ/δ-P_c complex and the transition from core/σ/δ-P_c to core/σ/δ-P_o, respectively.

The other possibility is that the release of delta is associated with the rate-limiting step in the reaction. In this case the unstable intermediate might be different from the intermediate in the core/sigma initiation pathway. The kinetic parameters K_{A*} and k_2 would refer to core/σ/δ-P_o and the conversion of core/σ/δ-P_o to core/σ-P_o.

Comparison of the sequences of the A2 and G2 promoters. There were two differences in the way that the RNA polymerase interacted with the A2 and G2 promoters: 1) the nucleotide requirements for heparin-resistant complex formation and 2) the effect of delta on complex formation. The sequences of the two promoters were compared 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 A2 and G2 promoters. The sequences of the two promoters and the E. coli/B. subtilis consensus sequence (Rosenberg and Court, 1979; Moran et al., 1982; Hawley and McClure, 1983) are shown in Figure 23.

The A2 promoter differs from consensus at three sites. One of the base alterations is at the highly conserved G residue at position 3 in the -35 region. Of the known (E. coli) promoter mutations, all of the promoters in which the G residue of the -35 region has been altered are "down" mutations (Hawley and 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 base alteration in the -35 region of the A2 promoter could therefore diminish promoter activity.

The G2 promoter has only two changes from consensus. One of these changes occurs at the second T residue in the -35 region. This base is very highly conserved in both the E. coli and Bacillus promoters (Moran et al., 1982; Hawley and McClure, 1983) but it is not clear how a base change at

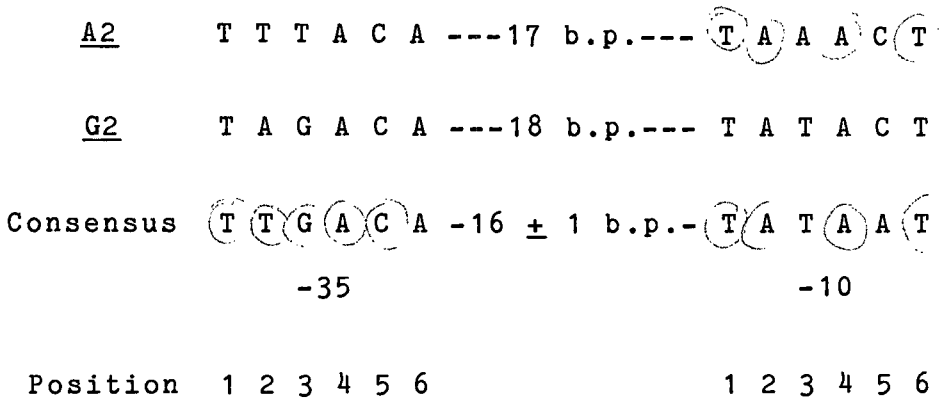


Figure 23. Promoter sequences of the A2 and G2 promoters (taken from Dobinson and Spiegelman, 1985 and Garvey et al., 1985). The sequences are compared with the consensus sequence (Rosenberg and Court, 1979; Moran et al., 1982; Hawley and McClure, 1983) for the promoters recognized by the Bacillus subtilis sigma-43 and E. coli RNA polymerase. The bases which are underlined in the consensus sequence are those which appear to be highly conserved in B. subtilis (Moran et al., 1982), and the bases in the A2 and G2 promoters which are changed from consensus are boxed.

this site would alter the promoter strength. The G2 promoter, which has an A residue at this position, is a fairly strong promoter and mutations at this site in E. coli promoters may either enhance or diminish promoter activity (Hawley and McClure, 1983).

The -10 regions of the two promoters are identical except for position 3. This site is only 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 has an A residue at that site. This base pair alteration could therefore be another factor in determining the relative strengths of the two promoters.

The two promoters also differ with respect to the length of the spacer sequence between the -10 and -35 regions. The spacer region of A2 is 17 basepairs long and the G2 spacer is 18 basepairs but, it is not clear how such a difference would alter the promoter function so profoundly. It is possible that the Bacillus RNA polymerase interacts more efficiently with promoters which have the longer spacer region, but such a conclusion cannot be made on the basis of only two promoters. Furthermore, the structure of the DNA which surrounds the promoter may also be important for promoter function.

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