

IN VIVO AND IN VITRO SYNTHESIS OF RIBOSOMAL RNA IN

BACILLUS SUBTILIS

by

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ABSTRACT

The work presented explored the in vivo and in vitro synthesis of ribosomal RNA in the Gram positive, spore-forming bacterium Bacillus subtilis. The investigation began with a study of rRNA synthesis in B. subtilis during steady state growth and under nutritional shift-up conditions. The percent of transcription which is ribosomal RNA was measured by hybridization of pulse labeled RNA to a specific DNA probe carrying the 3' end of the 23S RNA gene. The fractional rate of ribosomal RNA synthesis increased with cellular growth rate, and showed a rapid increase after a nutritional shift up. RNA synthesis during infection with an amber mutant of bacteriophage SP01 was also examined. Infected cells continued to synthesize rRNA at the preinfection rate, but could not respond to media enrichment by increasing the percent rRNA synthesis. The latter study suggested the existence of a specific RNA polymerase that transcribed ribosomal RNA genes.

The conclusions from the in vivo study led to an analysis of rRNA transcription in vitro. The isolation of the putative ribosomal RNA specific RNA polymerase was attempted by affinity chromatography on cellulose complexed with plasmid DNA containing the promoter region of the B. subtilis rrnB rRNA operon, and by sedimentation through a glycerol gradient. No difference in activity profile was observed when transcription activity at the rRNA tandem promoters was compared to activity at a non-ribosomal promoter.

Since in vivo analysis of the control of rRNA synthesis in Escherichia coli suggested that regulation occurs at the level of transcription initiation, in vitro transcription initiation at the B. subtilis rRNA promoters was investigated using the single round transcription assay. Initial rates of transcription were different at each of the two tandem promoters of the B. subtilis rrnB operon: the upstream promoter, P1, initiated slowly, while the downstream promoter, P2, initiated faster. In addition, transcription initiation at the two promoters appeared to be linked. The formation of a heparin resistant complex at the P1 promoter affected the stability of the heparin resistant complex formed at the P2 promoter. The kinetics of transcription initiation at the tandem rRNA promoters were examined using the tau plot analysis. RNA polymerase had a high affinity for both rRNA promoters, but the rate of initiation at these promoters was relatively slow when compared to non-ribosomal promoters. Finally, transcription initiation on two artificial tandem promoter constructs was compared with initiation on the native tandem promoter construct. In general, P1 was shown to have a positive effect on transcription from downstream promoters, but had specific effects on different promoters.

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LIST OF ABBREVIATIONS

| | |
|-----------|--|
| 2-ME | 2-mercaptoethanol |
| cpm | Counts per minute |
| EDTA | Ethylenediaminetetra-acetic acid |
| h | Hour |
| kb | Kilobase |
| min | Minute |
| moi | Multiplicity of infection |
| PFU | Plaque forming units |
| PMSF | Phenylmethylsulfonyl fluoride |
| SDS | Sodium dodecyl sulfate |
| SSC | 0.15 <u>M</u> NaCl, 0.015 <u>M</u> sodium citrate, pH 7 |
| TBE | 1 <u>M</u> Tris base, 1 <u>M</u> Boric Acid, 0.2 <u>M</u> EDTA |
| TCA | Trichloroacetic acid |
| u | growth rate |
| uL | microlitre |
| ug | microgram |
| <u>uM</u> | micro molar |

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I. INTRODUCTION

The ability of bacterial cells to grow and divide depends on many factors: the transport of nutrients from the surrounding medium, the conversion of those nutrients to accessible energy, and the use of that energy in the synthesis of macromolecules, such as RNA, DNA, polysaccharides, and protein. Under all growth conditions for Escherichia coli cultures, protein comprises at least half the cell mass (Bremer and Dennis, 1987). The engine of protein synthesis, the bacterial ribosome, is a complex organelle composed of 52 proteins (r-proteins) and three RNAs (rRNA). Schleif (1967) noted that under the most favorable laboratory conditions ribosomes can make up as much as 40% of the total dried cellular mass of the bacterium E. coli.

Over the last ten years the coordination of the synthesis of the ribosomal components in E. coli has been examined by a number of different groups (Maaloe, 1979; Nomura, et al., 1984; Lindahl and Zengel, 1986; Bremer and Dennis, 1987; Travers, 1987). It has been found that when rRNA molecules are unavailable for binding by r-proteins, certain r-proteins function as inhibitors of protein synthesis from their own messenger RNAs (Nomura, et al., 1984; Lindahl and Zengel, 1986). In other words, a primary rate limiting step in the synthesis of the ribosome, and hence protein synthesis and cell growth in general, is the synthesis of rRNA.

The work presented in this Ph. D. Thesis explored the in vivo and in vitro synthesis of rRNA in the Gram positive, spore-forming bacterium

Bacillus subtilis. In this introductory section I will first summarize the current understanding of global control of rRNA synthesis in bacteria, particularly E. coli. Second, I will briefly describe the structure of the operons which encode rRNA genes. I will then consider recent experiments which characterize the control of rRNA synthesis in both E. coli and B. subtilis. Since rRNA is the ultimate product from the rrn operons, that is, no translational amplification occurs, transcription initiation is thought to be a primary site of fine control; thus, I will discuss the current understanding of the stages of transcription initiation and the potential sites at which the initiation process can be controlled. I will conclude with a brief summary of the work presented in the body of the thesis.

A. Relationship between ribosome content and growth rate

The rate at which a bacterial cell grows dictates the rate of ribosome accumulation (Maaloe, 1979; Gausing, 1980). The number of ribosomes per cell for E. coli cultures growing over a range of different growth rates has been calculated and found to increase as the growth rate increases (Bremer and Dennis, 1987); that is, the number of ribosomes rather than the activity of individual ribosomes responds to changes in demand for increased protein synthesis. As I noted above, according to the current models for the control of ribosome synthesis, the synthesis of rRNA is probably the rate limiting step in the production of new ribosomes. The initiation rate at rrn genes has been calculated and found to increase from 4 initiations per minute per gene in cultures with a doubling time of 100 min to 61 initiations per minute per gene in cultures with a 24 min doubling time (Bremer and Dennis, 1987). What is

the mechanism which controls the rate of initiation at the rrn operons? Two basic models have been proposed: the feedback regulation model (Nomura, et al., 1984) and the direct regulation model (Bremer and Dennis, 1987).

Feedback regulation--The feedback regulation model proposes that rRNA synthesis is regulated by free, non-translating ribosomes which prevent the synthesis of ribosomes in excess of the amount needed for protein synthesis. The model makes a number of predictions which have been tested experimentally. The first prediction is that rRNA synthesis rates will not be significantly affected by changes in gene dosage. Experimental evidence has shown that an increase in copy number of complete rRNA operons resulted in a decrease in rRNA transcription from the individual rRNA operons while the overall rate of rRNA transcription was maintained at a constant level (Dennis, 1971; Jinks-Robertson, et al., 1983). The second prediction is that preferential inhibition of ribosome assembly will lead to a deficiency of free ribosomes which should cause a stimulation of rRNA synthesis. Using conditional mutants in ribosome assembly Takebe, et al. (1985) showed that rRNA synthesis increased under non-permissive conditions. While a body of indirect support for the feedback regulation model exists, a key prediction, namely, in vitro inhibition of rRNA synthesis by free ribosomes, has not been shown.

Direct regulation--The basic premise of the direct regulation model is that the synthesis of rRNA is controlled directly by RNA polymerase. The model postulates that the cellular pool of actively transcribing RNA polymerase is divided in two forms: form I which transcribes stable RNA

genes (rRNA and tRNA) and form II which transcribes all other genes. The the nucleotide guanosine tetraphosphate (ppGpp) is the suggested agent for the conversion between the two forms of RNA polymerase (Travers, et al., 1980; Ryals, et al., 1982). The strong inverse correlation of the intracellular concentration of ppGpp to the fractional rate of stable RNA synthesis (r_s/r_t) during steady state growth and nutritional shift-up conditions supports the more general role for this nucleotide in control of rRNA synthesis (Ryals, et al., 1982; Little, et al., 1983). Using well defined amber mutations, the beta subunit of RNA polymerase has been unambiguously shown to be a target for ppGpp (Glass, et al., 1986).

A synthesis of the models--Recent experimental evidence has suggested that the feedback regulation and the direct regulation models are not mutually exclusive. Cole and co-workers (1987) constructed a strain in which they could examine the effects of translation initiation factor IF2 limitation by placing the chromosomal copy of the gene encoding IF2 under lac promoter/operator control. Low concentrations of the lac inducer isopropyl thiogalactoside (IPTG) resulted in a significant decrease in growth rate, an increase in RNA content, and a large accumulation of non-translating ribosomes which were unable to cause feedback regulation of rRNA synthesis. The induction of IF2 synthesis led to a transient repression of rRNA synthesis before a new steady-state rate was attained. The authors suggest that actively translating, rather than non-translating, ribosomes lead to feedback regulation of rRNA. A possible interpretation of their results is that with the induction of IF2 synthesis, the non-translating ribosomes were able to initiate protein synthesis, which led to an increase in uncharged tRNA and the

subsequent production of ppGpp, and that this latter effect, in turn, caused the transient repression of rRNA synthesis.

B. Structure of ribosomal RNA operons

In E. coli four of the seven rrn operons are clustered near the origin of replication, while the other three are located within the first half of the chromosome to be replicated (Nomura, et al., 1984). In B. subtilis, clustering about the origin is even more pronounced: seven of the ten rrn operons are within 166 kb of the origin of replication (about 20% of the coding capacity of that region), while the other three operons are also located within the first half of the chromosome to be replicated (Widom, et al., 1988). While the most detailed analysis of the rRNA operon structure has been made in E. coli, no significant differences have been noted in the overall operon structure in B. subtilis (LaFauci, et al., 1986). Briefly, there is a tandem promoter region (see below), followed by a 171-173 base pair leader between the P2 promoter and the start of the mature 16S coding region. Between the 16S and the 23S genes is a 350-450 base pair spacer region which often contains 1 or 2 tRNA genes. The 23S gene is followed by the 5S gene. Distal to the 5S gene another 1 or 2 tRNA genes can be encoded.

C. Characterization of transcription from rRNA promoters

Many facets of transcription of rRNA operons have been extensively investigated. For example, Li et al. (1984) localized the antitermination system involved in E. coli rRNA synthesis. However, the regulation of antitermination is superimposed on the mechanism of growth rate

regulation; and as Gourse et al. (1986) have shown, the sequences which confer growth rate regulation are in the promoter region (see below). Thus, the experiments described in this section will focus on only two areas of research: experiments characterizing differential transcription from the tandem rRNA promoters, and experiments delineating non-promoter control sequences in the promoter region of the rRNA operon.

The tandem promoter arrangement has been defined as two (or more) promoters oriented in the same direction which transcribe the same gene or operon (McClure, 1985). Tandem promoters are found in the control regions of many bacterial operons that encode the components of the protein synthesizing system. The structure of these tandem promoters is such that each has near-consensus sequences at the -35 and -10 regions and the two start sites are separated by 70-120 bases (Lindahl and Zengel, 1986). The spacing between these tandem promoters theoretically allows the binding of an RNA polymerase molecule at each promoter site, and thus makes them different from other tandem promoters such as those at the gal operon in E. coli (Musso, et al., 1977; Aiba et al., 1981) or the veg promoters in B. subtilis (Le Grice and Sonenshein, 1982) which overlap and permit only one polymerase molecule in the promoter region. In E. coli greater than one third (6 out of 17) r-protein operons and all seven rRNA operons have a tandem promoter structure (reviewed in Lindahl and Zengel, 1986). In B. subtilis less is known about the structure of the promoter regions of r-protein operons, but nine of the ten rRNA operons have a tandem promoter arrangement (K. Bott and R. Rudner personal communication).

In E. coli the expression of the two rRNA promoters differs. Recombinant DNA techniques have been used to demonstrate that in vivo each of the two rRNA promoters is functionally distinct. Sarmientos and Cashel (1983) created a plasmid which contained the tandem promoter region of the rrnA operon fused to the terminator region of the rrnB operon to show that transcription from the upstream promoter (P1) increased exponentially with growth rate and predominated at high growth rates, and that transcription originating at the downstream promoter (P2) changed only slightly at different growth rates. They also demonstrated that upon carbon starvation, activity from P1 was not detectable but activity from P2 persisted. Sarmientos, et al., (1983) showed in another set of experiments using the same construct that in vivo transcription activity from the P1 promoter was subject to stringent control, while activity from the P2 promoter was inhibited by amino acid starvation in both wild type and relaxed strains. In a strain containing a plasmid in which the P2 region had been deleted, transcription from the P1 promoter showed stringent regulation. More recently, Gourse, et al. (1986) used lysogens containing various rrnB P1-lacZ gene fusions to show that growth rate regulation in E. coli takes place at the P1 promoter itself. Thus, in E. coli, the P1 promoter is growth rate regulated, stringently controlled, and predominant at high growth rates, while the P2 promoter is constitutively expressed.

The upstream promoter need not be the growth rate regulated one of the pair. Deneer and Spiegelman (1987), constructed plasmids which contained the B. subtilis rrnB tandem promoters or the separated rrnB promoters fused to the structural gene for tetracycline acetyltransferase. Using these constructs expressed in E. coli, they showed that

the downstream promoter (P2) was the growth rate regulated and transcriptionally more active of the B. subtilis rRNA tandem promoters, while the P1 promoter was constitutively expressed.

The canonical promoter elements at -10 and -35 of the stable RNA operons are not the only sites on the DNA that have an effect on the level of expression in E. coli. The upstream region between -98 and -40 of the tyrT promoter has been shown to be important for the high level of in vivo promoter activity. By following the production of a plasmid encoded tyrT-galK fusion transcript, Lamond and Travers (1985) showed that deletion of sequences in this region reduced the promoter strength to about 10% of the wild type level. A region upstream of the P1 promoter of the E. coli rrnB promoter has also been shown to affect in vivo transcription. Using a rrnB P1-lacZ fusion deletion series, Gourse, et al. (1986) demonstrated that the removal of sequences from -88 to -51 reduced transcription to about 5% of wild type.

D. In vitro analysis of transcription initiation

In vitro analysis of transcription initiation has been used to delineate possible mechanisms for the in vivo control of gene expression (McClure, 1985). A minimal model for RNA chain initiation has existed for many years (Walter, et al., 1967). Three major stages in transcription initiation have been defined and can be summarized as: 1) binding; 2) isomerization; and 3) promoter clearance (McClure, 1985). Results obtained with different promoters and with various techniques have been interpreted as demonstrating that any of these three stages

could be uniquely rate limiting. In the discussion that follows, R = RNA polymerase, D = DNA, and P = promoter.

Binding--The binding stage can be subdivided into at least two phases: 1) the binding of RNA polymerase to the DNA and 2) the location of a specific site along the DNA by the polymerase to form a closed complex.

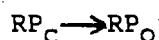


The binding constant, K_B , is the kinetic parameter used to describe this stage of transcription initiation (McClure, 1985). The rate at which enzyme binds to DNA is strongly dependent on the concentration of the reactants which is probably not a factor in vivo (Nomura, et al., 1986) and can be controlled in in vitro reactions so that neither is limiting. However, the second phase of the binding reaction could theoretically be rate limiting depending on the mechanism for the location of specific sites.

Each base pair on the DNA molecule is a potential non-specific binding site, and non-specific sites outnumber specific sites by many orders of magnitude. There is a limited number of specific binding sites on the DNA. If the mechanism for locating these sites was a three-dimensional diffusion process, the rate at which the interaction of polymerase with a specific site would be slow, and hence rate limiting (Chamberlin, 1974). However, if the locating mechanism involved linear diffusion along the contour of the DNA molecule, the search for a specific site would be facilitated due to a reduction in dimensionality

and/or a diminution in the volume to be searched (Singer and Wu, 1987). Wu, et al. (1983) have developed a rapid-mixing/photocross-linking technique and used it to directly follow the changing distribution of RNA polymerase along the DNA template as a function of time. Using this technique a set of kinetic traces which represent the transient occupation by polymerase of both specific and non-specific DNA-binding sites was produced and showed that linear diffusion along the DNA molecule must be playing a dominant role in promoter search (Singer and Wu, 1987). McClure (1985) noted that there was no evidence that diffusion to a promoter was slower than the subsequent steps in transcription initiation. More recently, Singer and Wu, (1987) reported the rate at which polymerase located a promoter as 50-fold faster than the reported rate of open complex formation at the lacUV5 promoter (S. Straney and Crothers, 1987).

Isomerization--Simply stated, the isomerization stage is characterized by the transition of the closed promoter complex to the open promoter complex:

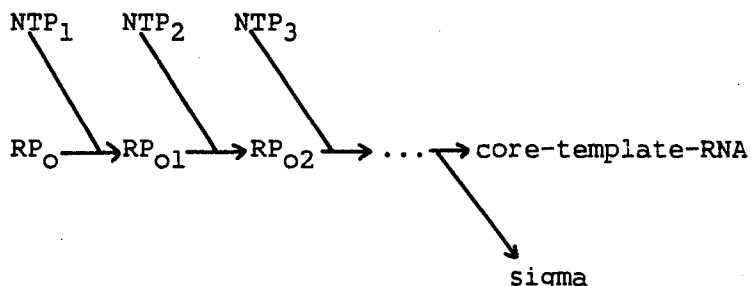


Extensive investigation of this transition, however, has shown it to be multi-stepped and probably unique for each promoter (McClure, 1985). The rate constant, k_f , is the kinetic parameter used to describe this stage of transcription initiation. After RNA polymerase locates a promoter, the initial complex formed is called the closed complex (Chamberlin, 1974). Since the closed complex is transient at 37° C, evidence for the existence of such a complex has been indirect and derived primarily from

analyses of the kinetics of chain initiation (Chamberlin, et al., 1982; Hawley, et al., 1982). Recently, however, it has been shown that polymerase specifically bound at a promoter at 0° C protects a reduced area from DNase I digestion when compared to footprints made at 37° C at the same promoter (Kovacic, 1987). Kovacic (1987) also noted that while the T7-A3 and the lacUV5 promoters had footprints of comparable size at 0° C, the protection patterns were unique for each promoter. It is known that formation of the open complex ultimately results in: 1) a topological unwinding of the DNA of 540° (Gamper and Hearst, 1982); 2) an increased exposure to chemical reagents ≥ 10 base pairs of DNA localized near the startsite (Siebenlist, 1979; Kirkegaard, et al., 1983); and 3) an alteration in the hyperchromicity of DNA bases (Hsieh and Wang, 1978; Riesbig, et al., 1979). McClure (1985) notes that the nature and magnitude of the structural changes in DNA or polymerase that precede these alterations in the template are unknown.

The formation of the open complex has been extensively studied with the lacUV5 promoter. Chemical, enzymatic, and electrophoretic methods have been used to identify two stable binary complexes at this promoter (S. Straney and Crothers, 1987; Spassky, et al., 1985). On the basis of kinetic studies, the form that predominated at lower temperatures has been proposed as an intermediate, the conversion of which is the rate limiting step, in the isomerization of closed to open complex formation (Buc and McClure, 1985). The proposed intermediate has been designated as "closed", since no single-stranded region was detectable in the DNA (Spassky, et al., 1985). Thus, the positioning of the enzyme at the promoter, rather than the unwinding of the template appears to be the rate limiting step in isomerization at the lacUV5 promoter.

Promoter Clearance--The promoter clearance stage of transcription initiation proceeds from the open complex through the formation of the first phosphodiester bond, to the dissociation of the sigma subunit from the ternary complex.



Subsequent to the tight binding of the polymerase to the promoter in the formation of the open complex, the enzyme must choose the precise transcription initiation point on the template, initiate the synthesis of an RNA chain, and escape from the stable open promoter complex to elongate the RNA. The overall process requires a balancing of two conflicting requirements: promoter binding should be tight in order to establish proper recognition, but not so tight that the polymerase is prevented from escaping the promoter to elongate the RNA.

The manner in which these two conflicting requirements is resolved at particular promoters has been examined by characterizing the production of abortive transcripts, and by following the release of the sigma subunit of RNA polymerase. DNase I footprints and methylation protection experiments have shown that RNA polymerase can remain tightly complexed at the lacUV5 promoter and reiteratively synthesize short RNA chains up to 10 bases long (Carpousis and Gralla, 1985; D. Straney and Crothers, 1987). Exonuclease III digestion through the upstream domains

of the lacUV5 promoter has demonstrated a slight loss of upstream open complex contacts during abortive transcription, but a large loss of these contacts upon escape from abortive cycling into productive transcription (D. Straney and Crothers, 1987).

In addition to the interaction between the polymerase and the upstream region of the promoter, the interaction between polymerase and the nascent transcript/template hybrid has been shown to be important in the rate at which the enzyme leaves the promoter. When inosine triphosphate was substituted for GTP in transcription initiation reactions at the lacUV5 promoter, the rate of the abortive transcript formation increased and the rate of productive transcript formation decreased (D. Straney and Crothers, 1987). The authors suggest that the weaker hydrogen bonding between the rI.dC base-pairs in the transcript/template hybrid favors the tight binding stage in the initiation reaction. Further support for the role of the downstream sequences has come from studies which monitor the point at which the sigma subunit is released from ternary complexes. Using a 5'-azide photoreactive dinucleotide in transcription initiation reactions, Bernhard and Mearse (1986) reported that the sigma subunit was labeled by RNA chains 9-13 bases long at the lambda P_R promoter and by chains 3 bases long at the T7 A1 promoter. The authors suggest that early sigma release may occur when an alternating pattern of hydrogen bond donors and acceptors is observed in the RNA/DNA hybrid as is the case at the T7 A1 promoter, but not at the lambda P_R promoter. Thus, the promoter clearance stage in transcription initiation appears to be rate limiting for the lambda P_R and the lacUV5 promoters, but not limiting for the T7 A1 promoter.

Transcription initiation in B. subtilis--Transcription initiation has been studied almost exclusively at a few E. coli promoters. Recently, however, initiation has been investigated at two $\phi 29$ promoters used by the predominant B. subtilis sigma⁴³ RNA polymerase (Dobinson and Spiegelman, 1985 & 1987). The kinetic parameters, K_A^* (K_B) and k_2 (k_f), were determined for the $\phi 29$ A2 promoter and found to be characteristic of weak E. coli promoters, in that these parameters were similar to those reported for three lac mutants (Dobinson and Spiegelman, 1985; Stefano and Gralla, 1982). The effect of the delta subunit of B. subtilis polymerase on transcription initiation at the weak $\phi 29$ A2 promoter and the strong $\phi 29$ G2 promoter was compared (Dobinson and Spiegelman, 1987). It had been proposed that delta played a role in enhancing the specificity of polymerase binding by inhibiting the formation of stable complexes at nonspecific sites on the Bacillus chromosomal DNA (Achberger, et al., 1982; Doi, 1982). Dobinson and Spiegelman (1987) found that delta inhibited the formation of heparin resistant complexes at the A2 promoter but not at the G2 promoter. To account for these differences the authors suggested that the release of delta from the A2-polymerase complex might be the rate limiting step in the initiation reaction at that promoter.

E. Summary of the work presented in this thesis

This investigation began with a study of rRNA synthesis in B. subtilis during steady state growth and under nutritional shift-up conditions. I demonstrated that in B. subtilis the fractional rate of rRNA synthesis increased as a function of growth rate, and was similar to that reported for E. coli. I also examined the relationship between rRNA

synthesis and RNA polymerase availability using an amber mutant of the SP01 phage. Evidence was found which suggested the existence of a ribosomal specific RNA polymerase.

The conclusions from the in vivo study led to an analysis of rRNA transcription in vitro. The isolation of the putative ribosomal RNA specific RNA polymerase was attempted, but no difference in activity profile was observed when transcription activity at the rRNA tandem promoters was compared to activity at a non-ribosomal promoter. In vivo analysis of the control of rRNA synthesis in E. coli suggested that regulation occurs at the level of transcription initiation, therefore, in vitro transcription initiation at the B. subtilis rRNA promoters was investigated using the single round transcription assay. I showed that the formation of a heparin resistant complex at the P1 promoter affected the stability of the heparin resistant complex formed at the P2 promoter. The kinetics of transcription initiation at the rRNA promoters were examined and I demonstrated that RNA polymerase had a high affinity for both rRNA promoters, but the rate of initiation at these promoters was relatively slow when compared to non-ribosomal promoters. Finally, transcription initiation on two artificial tandem promoter constructs was compared with initiation on the native tandem promoter construct. In general, P1 was shown to have a positive effect on transcription from downstream promoters, but had specific effects on different promoters.

II. MATERIALS AND METHODS

A. Bacterial strains, Phage, and Plasmids

1. Bacterial strains--Bacillus subtilis 168M (obtained from H. R. Whiteley, University of Washington, Seattle, WA.) was the host for bacteriophage SP82 infections and the source of purified RNA polymerase. B. subtilis L15 (su⁺³, thr) (Georgopolous, 1969) was obtained from W. D. Ramey, University of British Columbia and served as the host for bacteriophages SP01 amber 34 and ϕ 29. Escherichia coli SF8 (Olson, et al., 1977) was the host for plasmids pVG-1, p328-5, and pHD1.8. E. coli HB101 was used as the host for plasmids pKK427B, pTLXT210, pTLXT3B, pPldP2 and pPlA2. The E. coli strain containing p12E2 (Moran and Bott, 1979), a pBR313 recombinant plasmid containing portions of B. subtilis ribosomal RNA genes, was provided by K. Bott (University of North Carolina, Chapel Hill).

2. Phage--Bacteriophage SP82 particles were purified by concentrating 4 L of phage lysate by overnight precipitation with polyethylene glycol 6000 (to 10%, w/v) and NaCl (to 1.9%, w/v) at 4°C. The precipitate was harvested by centrifugation, resuspended in 20 mL of 20 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, and 0.1 M NaCl, and dialyzed against 4 L of the same buffer for 48 h at 4°C. The dialyzed suspension was centrifuged at 27,000 x g for 10 min and the supernant liquid was stored at 4°C. Titre of the supernant liquid was approximately 2 x 10¹¹ (PFU)/mL.

Bacteriophage SP01 amber 34 (Fujita, et al., 1971 and Fox, 1976) particles were purified as described above with the following change: after dialysis of the resuspended polyethylene glycol 6000 precipitate, 0.8 g CsCl was added per mL of phage suspension and the suspension centrifuged at 32,000 rpm at 20°C for 24 h in a Beckman 50 Ti rotor. The phage band was removed and dialyzed against 2 x 4 L 20 mM Tris (pH 7.4), 1 mM MgCl₂, and 0.1 M NaCl for 16 h at 4°C. The purified phage particles were stored at 4°C. Titre of the dialysate was 1.5×10^{12} PFU/mL on the suppressor host and 2.4×10^6 PFU/mL on the wild type host.

Bacteriophage ϕ 29 particles were purified as described for the SP01-like phages with the following changes: phage particles were concentrated from infected culture lysates in 11% w/v polyethylene glycol 6000 and 2.3% w/v NaCl, and 0.73 g CsCl was used per mL phage suspension in the gradient centrifugation step. For bacteriophage ϕ 29 DNA, purified phage particles were treated with 1% SDS, 20 mM EDTA and heated at 65°C for 10 min. The ruptured particles were then treated with 50 ug/mL Proteinase K (Sigma) for 2 h at room temperature. The DNA was purified by phenol extraction and ethanol precipitated in the presence of 3% sodium acetate. The DNA was dissolved in 0.1x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH7) and stored over chloroform.

3. Plasmids--Plasmid DNA was purified using the Clewell and Helinski (1972) cleared lysate method with the modifications described by Dobinson and Spiegelman (1985). Plasmid DNA was purified from the cleared lysate by centrifugation in CsCl-ethidium bromide. The isolated plasmid was treated with butanol to remove the ethidium bromide, ethanol precipitated and redissolved in distilled water or 0.1x SSC.

B. Media and Growth Conditions

The media used in the B. subtilis growth rate experiments were of two basic types. Defined medium plus L-tryptophan (DMT) is similar to that described by Hiatt and Whiteley (1978) except L-tryptophan (Sigma) (to 5 ug/mL) and 0.5% (w/v) casamino acids (Difco) were used. Various carbon sources (glucose to 0.1%, sodium acetate to 0.25%, and succinic acid to 0.25%) and nutrients (casamino acids to 2%, beef extract (Oxoid) to 0.5%, adenosine (Sigma) to 0.6 mg/mL, uridine (Sigma) to 0.6 mg/mL, thymidine (Sigma) to 0.6 mg/mL, and a vitamin mixture described by Rodin (1972)) were added to DMT. Three types of complex media were used: trypticase soy (Difco), 2.5 g/L, with glucose to 0.1%; L broth as described by Silverman and Simon (1973); and M medium (Yehle and Doi, 1967) modified as follows (per litre): 2.5 g yeast extract (Difco), 5 g tryptone (Difco), 10 g NaCl, and 1 g glucose. After sterilization all media were adjusted to final concentrations of 5 mM MgSO_4 and 0.02 mM MnCl_2 . All B. subtilis cultures were grown at 37°C unless otherwise stated. B. subtilis L15 when grown as a host for $\phi 29$ and B. subtilis 168M when grown as a source for RNA polymerase were grown in unmodified M medium (Yehle and Doi, 1967). Doubling time was determined by change in optical density at 640nm, and growth rate (u) was calculated by dividing the natural logarithm of 2 by the doubling time (g) in hours:

$$u = (\ln 2 / g)$$

E. coli strains bearing plasmids were grown at 30°C in M9 medium (Champe and Benzer, 1962) modified as follows in final concentrations:

0.25% glucose, 0.2% casamino acids, 0.1 mM FeCl₃, 0.1 mM CaCl₂, 200 ug uridine/mL, 10 ug thymidine/mL, and 20 ug thiamine/mL.

C. Bacteriophage SP82 Burst Assays

Cultures were grown on a rotary platform shaker set at 250 rpm. Initial medium volume per flask was 100 mL per 500 mL flask. The phage infections were performed as described by Palefski, et al., (1972) with the following alterations. Cultures were inoculated with 1.5 mL from a standing overnight culture grown in DMT, grown to a density of 3.9×10^8 cell/mL and then infected at a multiplicity of 1.6 phage/cell. Burst size was determined as the ratio of phage produced/mL to infective centers/mL. The phage yield was measured by plating for PFU immediately after the optical density drop in the infected culture indicated cell lysis. Infective centers were determined by removing 1 mL of culture 1 min after infection, centrifuging for 4 min in an Eppendorf centrifuge, removing the supernant liquid, resuspending the pellet in 1 mL of M medium, and plating at 10^{-5} for plaques.

D. Transport Assays

Parallel cultures of B. subtilis were grown in DMT with casamino acids (to 0.2% or 0.01%) and acetate or succinic acid (to 0.2%) as carbon source to a density of 5×10^7 cells/mL. One of the cultures was infected with SP01am34 at a multiplicity of infection of 25 and growth of both cultures continued for a further 10 min. Uptake assays were performed as described by Beaman, et al. (1983) with the following changes: radioactive nutrient (1-2.5 uCi/mL of culture) was added to

each culture and 0.2 mL samples were taken 20 sec after the addition of labeled nutrient and then every 45-60 sec for upto 3 min. Samples were filtered through nitrocellulose membrane filters (Millipore), the membrane washed 3 times with cold medium, dried and counted in a Beckman Scintillation Counter. Immediately prior to radioactive nutrient addition, duplicate culture samples were taken for protein determination (Sandermann and Strominger, 1972). In all cases, except for the glucose assays, the labeled nutrient was added directly to the culture medium and no attempt was made to calculate the final nutrient concentration. When this procedure was tried for the glucose assays, erratic results were obtained. The undiluted concentration of the glucose label was 0.019 μM while the reported K_m for glucose in a wild type strain of B. subtilis is 0.01 mM (Price and Gallant, 1983), therefore, the labeled glucose was added in a 0.23 mM glucose solution to give a 3 μM final glucose concentration.

E. Preparation of pulse labeled RNA

B. subtilis was grown in M medium or in defined medium plus various carbon sources (see above) to a density of 5×10^7 cells/mL. In shift-up experiments, the culture was enriched with 1/10 volume of 10x M medium or a mixture of 19% glucose, 10x vitamins (Rodin, 1972). Unshifted cultures were labeled with 10 μCi [5,6- ^3H]Uridine per mL culture while the shifted cultures were labeled with 200 $\mu\text{Ci/mL}$. SP01am34 phage were added to a multiplicity of infection of about 25. Labeling was stopped by the addition of sodium azide to 0.05 M and rapid cooling to 0°C . RNA was isolated from 1 mL aliquots according to the freeze-thaw method of Sogin, et al. (1977) except 100 $\mu\text{g/mL}$ yeast tRNA was included in the buffers as

carrier. After SDS-EDTA treatment the samples were phenol extracted twice, ethanol precipitated, ethanol washed, resuspended in 0.2 mL sterile distilled water and stored at -20° C.

F. Construction of pVG-1

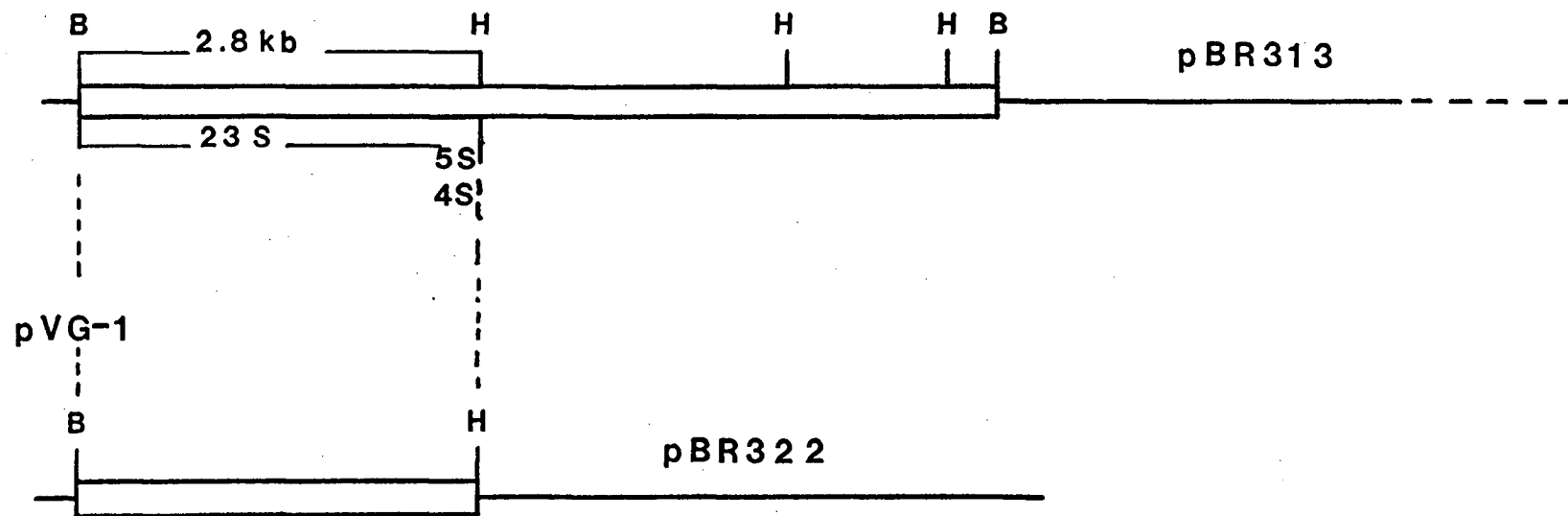
The hybridization probe used in the in vivo studies, pVG-1, contained sequences from the genes encoding the 23S and 5S rRNAs of B. subtilis isolated from p12E2 (Moran and Bott, 1979). Plasmid pVG-1 was constructed by cleaving p12E2 with HindIII and BamHI, and isolating the 2.8 kb fragment by electroelution after acrylamide gel electrophoresis. The isolated fragment was ligated to appropriately cleaved pBR322 DNA and after transformation colonies resistant to ampicillin and sensitive to tetracycline were scored. Plasmid pVG-1 was verified as having the correct DNA fragment by hybridization to the parent plasmid, hybridization to in vivo labeled rRNA, and by restriction enzyme analysis (data not shown). Figure 1 shows a restriction enzyme map of the parent plasmid (taken from Moran and Bott, 1979) and the DNA fragment subcloned into pVG-1. Restriction endonucleases and DNA ligase were obtained from New England Biolabs and used according to the supplier's instructions.

G. Hybridization assays

It was observed that after heat denaturation plasmid DNA would "snap-back" as monitored by rapid decrease in OD_{260} . It was found that plasmid DNA which had been knicked with S1 nuclease (Lilley, 1980), using 1 unit of enzyme per 10 μ g DNA, remained denatured. Denatured DNA was immobilized on nitrocellulose membrane filters (Schleicher and Schuell)

Figure 1. Construction of plasmid pVG-1. A DNA fragment 2.8 kb long from plasmid p12E2 delineated by BamHI (B) and HindIII (H) restriction endonuclease sites was isolated and cloned into pBR322 treated with BamHI and HindIII. The map of plasmid p12E2 is from Moran and Bott (1979). Approximate positions of the 23S RNA (23S), 5S RNA (5S), and tRNA (4S) coding regions are shown. Open bars represent cloned B. subtilis DNA, and solid line represents vector DNA.

p12E2



as described by Gillespie and Spiegelman (1965). Hybridization experiments were performed at constant RNA concentration with increasing DNA concentration in 6x SSC, 0.1% SDS at 67°C for 16 h. After hybridization filters were washed in 6x SSC at 67°C, treated with 10 ug/mL RNase A (Sigma) at 37°C for 30 min, and washed in 6x SSC at 67°C two more times.

H. Calculation of the percent in vivo rRNA synthesized

Conversion of the radioactivity bound to pVG-1 DNA filters to the rate of rRNA synthesis included the following: (1) the hybridization efficiency (determined to be 0.88 using purified ^3H -rRNA); (2) the size of the hybridization probe (53% of the rRNA transcription unit, Moran and Bott, 1979); and (3) the fact that the uridine content of B. subtilis rRNA (20.7%, Doi and Igarishi, 1964) differs from the total thymidine content of B. subtilis DNA (28.5%, MacHattie and Thomas, 1968) and from the hydroxymethyl uridine content of SPO1 DNA (28%, Szybalski, 1968). In addition, three assumptions were made: (1) labeled uridine entered mRNA and rRNA precursor pools equally; (2) uridine was not involved in cytidine metabolism; and (3) the uridine content of non-rRNA was equal to the thymidine content of the total DNA.

The synthesis of rRNA (the percent rRNA) was monitored by the ratio of nucleotides incorporated into rRNA (C_r) to the total nucleotides incorporated (C_T) in the one min pulse labeling interval or,

$$\% \text{ rRNA} = [(C_R^*) / (C_T^*)] \times 100\%$$

The values of C_R^* and C_T^* were calculated from the total radioactivity input in a hybridization and the resulting radioactivity bound to pVG-1 DNA, correcting for the hybridization efficiency and probe size in the case of C_R^* and the total uridine content of the RNA in the case of both C_R^* and C_T^* . If C_R cpm of pulse labeled ^3H -RNA, out of total input of C_T cpm, bound to pVG-1 filters, then

$$C_R^* = \frac{C_R}{0.53 \times 0.88 \times 0.207}$$

and

$$C_T^* = C_R^* + \left[\frac{C_T - \left(\frac{C_R}{0.88 \times 0.53} \right)}{0.28} \right]$$

Note the specific activity of the uridine label was not included as it cancels out of the percent rRNA calculation.

I. pHD1.8 DNA-Cellulose Column

A pHD1.8 DNA-cellulose column for isolation of an rRNA specific RNA polymerase was prepared using a modification of the Litman (1968) procedure. Briefly, 830 mg of washed cellulose (undesiccated) was mixed with 2.75 mL of purified pHD1.8 DNA 564 ug/mL, dried 16 h in a vacuum

desiccator and lyophilized a further 16 h in a freeze drier. Four hundred ninety milligrams were recovered, suspended in 30 mL absolute ethanol, and irradiated with long wave ultraviolet light for 20 min. The material was washed with 250 mL 0.1 M NaCl and dried for three days in a vacuum desiccator. Four hundred seventy milligrams were recovered. The pHD1.8 DNA-cellulose was suspended in 10 mL of buffer containing 10% glycerol, 0.01 M Tris-HCl pH 7.9, 1 mM EDTA, 0.01 M MgCl₂, 50 ug/mL PMSF, 0.2 M 2-ME, 0.05 M NaCl, poured into a 5cc syringe, and washed with 75 mL of the same buffer.

J. Purification of B. subtilis holoenzyme

1. RNA polymerase for single round transcription assays was purified from freshly grown B. subtilis 168M as described previously (Dobinson and Spiegelman, 1985) with the following modifications. The 0.6 M NaCl DNA cellulose fractions containing RNA polymerase activity were pooled and subjected to chromatography on heparin-sepharose (Dobinson and Spiegelman, 1985). The 0.6 M NaCl fractions containing enzyme activity were pooled, concentrated and sedimented through the 15-30% glycerol gradient described by Dobinson and Spiegelman (1985). The peak enzyme fractions from the glycerol gradient were pooled and concentrated by chromatography on a second 0.5 mL heparin-sepharose column. The protein concentration was determined (Sandermann and Strominger, 1972) using bovine serum albumin (Fraction V, Sigma Chemical Co.) as the standard. Total enzyme activity was assayed by as described by Spiegelman and Whiteley (1974), with several modifications. O29 DNA was incubated in the presence of 0.04 M Tris-HCl pH 7.9, 0.02 M MgCl₂, 0.05 M NaCl, with 0.08 mM GTP, CTP, and ATP, and 8 uM UTP and 25 uCi

[³H]-UTP for 2 min, 10 uL of enzyme was added and incubation continued for a further 10 min. 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.). The amount of active enzyme in the purified preparation was found to be 50% as measured by specific initiations on the ϕ 29 G2 promoter as described by Brion and Spiegelman (in preparation).

2. RNA polymerase for rRNA specificity studies was purified from B. subtilis 168 (Iowa Grain Processing Corp.) as described above. One and a half millilitres of the pooled 0.6 M NaCl fractions from the first heparin-sepharose chromatography were diluted with a buffer containing 10% glycerol, 0.01 M Tris-HCl pH 7.9, 1 mM EDTA, 0.01 M MgCl₂, 50 ug/mL PMSF, 0.2 M 2-ME to 0.05 M NaCl (monitored on a Bach-Simpson Ltd. type CDM-2f conductivity meter). The sample was loaded on a 1.5 cc PHD1.8 DNA-cellulose column and eluted with a 10 mL 0.05-0.8 M NaCl gradient of the same buffer. Four drop fractions were collected and assayed for RNA polymerase activity as described previously (Dobinson and Spiegelman, 1985). Fractions containing peak activity were used in a standard transcription assay with PHD1.8 (rrnB tandem promoter) or p328-5 (ϕ 29 A2 promoter) as template to assess enzyme specificity. The sodium chloride concentration of the fractions was determined by establishing a standard curve of conductivity vs NaCl concentration, and measuring the conductivity of a 1/20 dilution of each fraction.

K. Transcription Assays

1. Standard Assay--Transcriptions were done in 100 μ L of buffer containing 40 mM Tris-HCl pH 7.9, 20 mM $MgCl_2$. For assays of chromatography fractions of NaCl gradient elutions, all reactions were made up to 60 mM NaCl. ATP, GTP, CTP, and UTP were all 400 μ M except when the transcripts were labeled, in which case the labeled nucleotide was at 10 μ M. Reactions where pHD1.8 served as template contained 5 uCi of [α - ^{32}P]ATP (New England Nuclear), while reactions in which p328-5 was the template contained 4.5 uCi [α - ^{32}P]UTP (Amersham). Reactions were started by the addition of the nucleotide mix to a solution of DNA and RNA polymerase which had been incubated for 5 min at 37 $^{\circ}$ C. Transcription was carried out for 10 min and stopped by with 25 mM EDTA and chilled on ice.

2. Single Round Transcription Assay-- The single round transcription assay was performed as described previously (Dobinson and Spiegelman, 1987). Briefly, linearized template DNA and RNA polymerase were incubated together in the presence of two or three nucleoside triphosphates (400 μ M final concentration). At times indicated, 38 μ L of the reaction were removed and added to a 2 μ L mixture containing heparin (5 μ g/mL final concentration) and remaining nucleoside triphosphates, one of which was the labeled nucleotide and present at 10 μ M. After 10 min to allow elongation, reactions were stopped by the addition of 10 μ L of 10 M urea, 0.1 M Trizma Base, 0.1 M boric acid, and 2 mM EDTA. Half of each reaction was electrophoresed through an 8% polyacrylamide gel containing 7 M urea at 35 volts/cm. Regions of the gel containing specific transcripts were localized by autoradiography, excised, and the

amount of incorporated label determined by measuring the Cerenkov radiation in each slice. The number of transcripts was calculated by dividing the Cerenkov radiation in such gel pieces (counts per min) by the specific activity of a single transcript (the specific activity of the labeled nucleotide in the reaction corrected by the number of times the nucleotide occurs per transcript). The ribonucleoside triphosphates used were isolated as nucleoside triphosphates (97-99% pure, Sigma Chemical Co), it was found that the pyrimidine nucleotides formed by the enzymatic phosphorylation of 5'-CMP or uridine contained levels of ATP which permitted the formation of full length transcripts in the absence of added ATP.

III. IN VIVO RESULTS

One of the purposes of the experiments reported in this section was to examine shift-up induced synthesis of rRNA in cells which had been depleted of their pools of RNA polymerase. The use of restrictive conditions for conditional mutants or RNA polymerase inhibitors has been shown to change the states of ribosome and RNA polymerase and thereby alter the transcriptional activity of the cell (Yura and Ishihama, 1979; Lindahl and Zengel, 1982). It was, therefore, considered important that the cells be depleted of their pool of excess polymerase without changing the transcriptional activity in the cell.

The Bacillus phage SP01 was used to carry out depletion of the cellular pool of RNA polymerase. Phage SP01 and SP82 (a closely related phage) are large, hydroxymethyl uracil (HMU) containing DNA phages of Bacillus. Infection with these phages leads to specific and temporal modifications of the cellular RNA polymerase (Doi and Wang, 1986). The first modification, occurring early in the lytic cycle, involves the replacement of the major host sigma subunit, sigma⁴³, with a phage encoded subunit, gp28, (Fujita, et al., 1971; Fox, 1976; Duffy and Geiduschek, 1975). The second replacement occurs later in the infection cycle and involves phage gene products gp33 and gp34 (Fujita, et al., 1971; Fox, 1976; Tjian and Pero, 1976). In vivo replacement of sigma⁴³ by gp28 is accompanied by a 95% decrease in host mRNA synthesis indicating that most bacterial promoters are no longer utilized (Gage and Geiduschek, 1971; Geiduschek and Ito, 1982). Phage with amber mutations in the gene for gp34 do not synthesize late proteins or DNA but do

undergo the first polymerase modification and the shut-off of host mRNA synthesis (Fujita, et al., 1971; Fox, 1976). Thus, by infecting B. subtilis with SP01am34, depletion of much of the cellular RNA polymerase pool could take place without inhibiting transcription.

The results presented in this section are divided into three parts:

1) preliminary studies in which the interaction of B. subtilis culture conditions and bacteriophage SP01 infection were investigated; 2) control studies which established the levels of rRNA synthesis in B. subtilis during steady-state growth and in shift-up conditions; and 3) experimental studies where the rRNA synthesis in SP01am34 infected cultures was examined. The overall theme of these experiments was the interaction between rRNA synthesis and RNA polymerase availability.

A. Interaction of B. subtilis culture conditions and SP82 phage infection

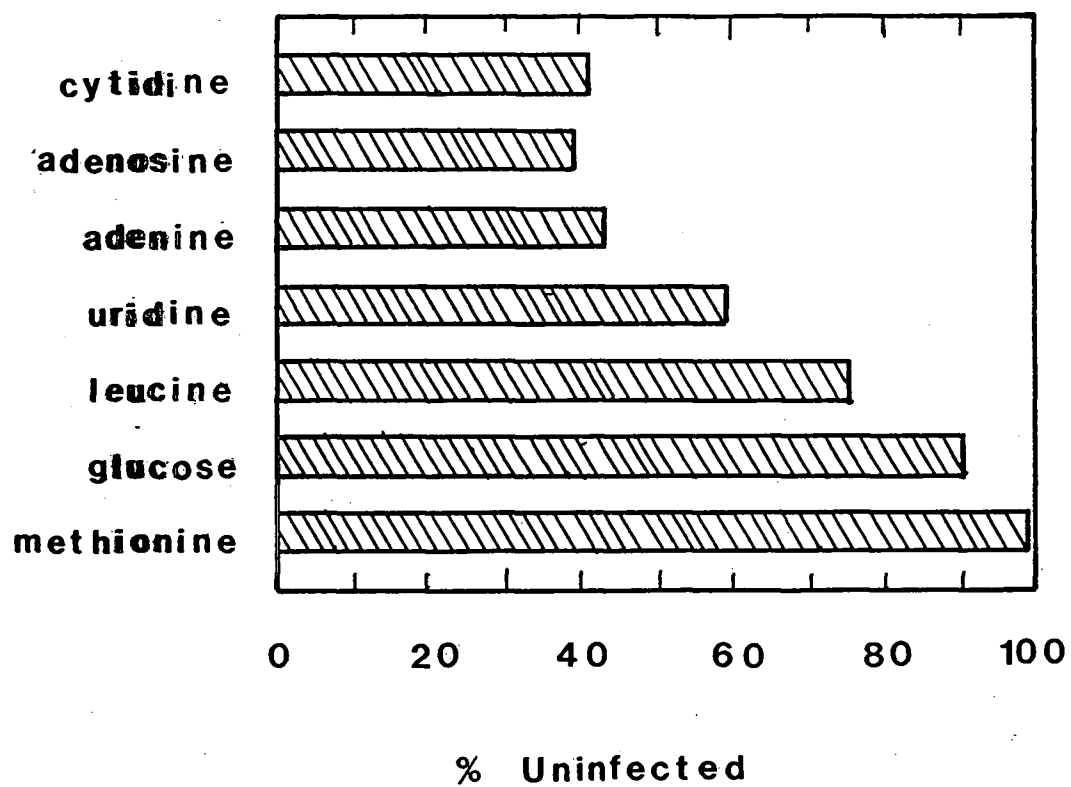
1. Effect of SP01 phage infection on the rate of nutrient transport. If infection with SP01 was going to be used to deplete the host pool of RNA polymerase prior to a nutritional shift-up, then the effect of phage infection on the host's ability to transport the nutrients needed to be known. The following set of experiments was not intended to represent an in depth analysis of nutrient transport in B. subtilis (see Beaman, et al. 1983), but rather a brief study investigating a particular set of experimental conditions.

Parallel cultures of B. subtilis were grown in DMT with acetate or succinic acid as carbon source and casamino acids to a density of 5×10^7

cells/mL. One of the cultures was infected with SP01am34 and growth of both cultures continued for a further 10 min. Radioactive nutrient (1-2.5 uCi/mL of culture) was added to each culture and 0.2 mL samples were taken 20 sec after the addition of labeled nutrient and then every 45-60 sec for up to 3 min. Samples were filtered on to nitrocellulose membrane filters, the membrane washed 3 times with cold medium, dried and counted. Immediately prior to radioactive nutrient addition, duplicate samples were taken from each culture for protein determination (Sanderman and Strominger, 1972).

SP01am34 infection did not affect the rates of transport of the nutrients tested equally (Figure 2), suggesting that control of nutrient transport in B. subtilis is complex. The rate of methionine transport in the phage infected cultures was unaffected, and the rates of glucose and leucine transport were still 90% and 75%, respectively, of the control. The rates of nucleoside and base transport were more dramatically affected by phage infection. The rates of adenine, adenosine, and cytidine transport in the infected cultures were reduced to approximately 40% of the control, while the rate of uridine transport was reduced to 60% of the control. It is not clear from these single concentration experiments whether the K_m or the v_{max} or both were affected by phage infection. Beaman et al. (1983) reported the apparent K_m for adenosine uptake by B. subtilis as 12 μM . In the shift-up experiments reported below the final adenosine concentration was about 2 mM ; if the apparent K_m for adenosine uptake was increased by 60%, the substrate binding sites would still be saturated at this concentration. Although the reduction in the rates of nucleoside and base transport were of interest, it was more significant for the subsequent shift-up studies that phage

Figure 2. Effect of SP01am34 infection on nutrient transport. The histogram shows transport rates for seven nutrients in an SP01am34 infected culture relative to the rates in uninfected cultures. Parallel cultures of B. subtilis were grown in DMT + 0.25% acetate or 0.25% succinate and 0.2% casamino acids to a density of 5×10^7 cell mL⁻¹. One of the cultures was infected with an moi=25 and growth of both cultures continued for a further 10 min. Radioactive nutrient (1-2.5 uCi/mL) was added to each culture. Samples (0.2 mL) were taken 20 sec after the addition of labeled nutrient and then every 45-60 sec for up to 3 min. Samples were filtered on to nitrocellulose membranes, the membranes washed 3 times with cold medium, dried and counted. Immediately prior to radioactive nutrient addition, duplicate samples were taken from each culture for protein assay. The rate of transport per mg protein (cpm/min/mg protein) was determined for both cultures. Data are presented as the rate of transport in infected cultures divided by the rate of transport in uninfected cultures x 100 to give the % Uninfected.



infection did not markedly reduce the rates of glucose or amino acid transport.

2. SP82 burst size is dependent on growth rate. In preliminary experiments to determine whether cells grown in poor media could support successful SP82 infection, it was noticed that the burst size of wild type phage varied dramatically with the growth rate of the host. To further investigate this observation the SP82 burst size was measured over a range of bacterial growth rates. It was found that there was a linear relationship between the natural logarithm of the burst size and the cell growth rate.

The media used for growth of the bacterial cultures and the growth rates obtained with each medium are listed in Table I. For some media a range of growth rates is reported. This range reflects day to day variations during repeat experiments. One hundred millilitre cultures were inoculated with 1.5 mL from a standing overnight culture grown in DMT. Cultures were grown to a density of 3.9×10^8 cell/mL, and then infected at a multiplicity of 1.6 phage/cell. Burst size was determined as the ratio of phage produced/mL to infective centers/mL. Since plating for phage burst and infective centers were performed within 50 min of each other, equal plating efficiencies for the two determinations were assumed.

In relating burst size and growth rate, the possibility was considered that at high growth rates the phage lytic time might be shortened and that the large yields were caused by multiple rounds of infection. To investigate this possibility, a one-step growth experiment utilizing a

TABLE I

Growth rates for B. subtilis in various media

| <u>Medium</u> | <u>Growth Rate</u> |
|---|--------------------|
| 1. M | 1.46-2.97 |
| 2. L Broth | 2.81 |
| 3. Trypticase soy | 1.47 |
| 4. DMT + 0.1% glucose | 1.34-1.43 |
| 5. DMT + 0.25% succinic acid | 0.76-1.09 |
| 6. DMT + 2% casamino acids | 0.59-0.76 |
| 7. DMT + 0.5% beef extract | 1.39 |
| 8. DMT + 0.5% beef extract + 0.1% glucose | 1.97 |
| 9. DMT + 0.1% glucose + vitamins ^a + adenosine ^b + uridine ^b | 1.79-1.98 |
| 10. DMT + 0.1% glucose + vitamins ^a + adenosine ^b + uridine ^b + thymidine ^b | 2.30 |

^a As described by Rodin (1972).

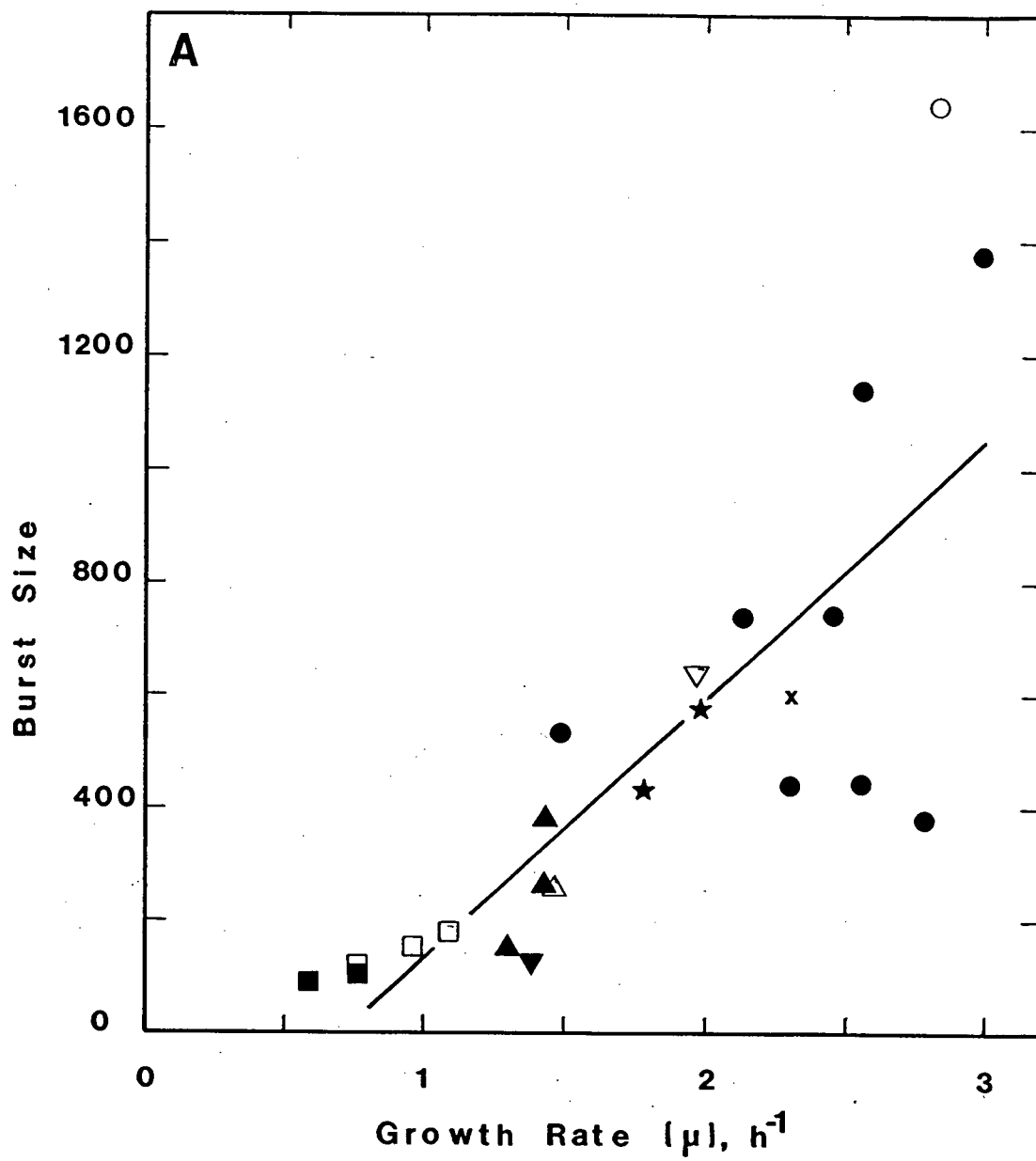
^b 0.6 mg/mL.

culture of bacteria grown in M medium was performed (data not shown). The culture had a doubling time of 19 min and burst size of 1200, and exhibited a single increase of 10^2 PFU/mL over a span of 5 min, with a midpoint of 38 min, the lytic time previously reported (Lawrie, et al., 1976). Furthermore, the time for infected cultures to reach minimum optical density ($OD_{640}=0.05$) was about the same (45-50 min) for all growth rates. Thus, it is unlikely that multiple rounds of phage infection had taken place.

When the burst size was plotted against the growth rate the relationship was markedly nonlinear (Figure 3a) having a linear correlation coefficient of 0.79 (by the method of least squares). When the natural logarithm of the burst size was plotted against the growth rate (Figure 3b) a more linear relationship, with a correlation coefficient of 0.88, was observed. It is obvious that the burst size is growth rate dependent. The results obtained with a single medium (for example, medium 1) exhibit the same trend as seen overall, suggesting that the variation in burst size was indeed growth rate dependent rather than medium dependent. Schaechter, et al. (1958) reported that for Salmonella cellular contents of DNA, RNA, and protein at a given temperature depended only on growth rate and not on medium composition.

It is significant that a logarithmic function better describes the relationship between the burst size and growth rate. Leduc, et al. (1982) measured the accumulation of total RNA and RNA polymerase in B. subtilis over a range of growth rates similar to those shown in Figure 3, and found that both total RNA per cell and the percent active RNA polymerase increased exponentially with growth rate as has been reported

Figure 3. Burst size of SP82 as a function of host cell growth rate (μ). A. The phage burst size is plotted against the cell growth rate. The line has a regression coefficient of 0.79. B. The natural logarithm of the phage burst is plotted against the cell growth rate. The line has a regression coefficient of 0.883. The symbols shown refer to media listed numerically in Table I: ●, medium 1; ○, medium 2; △, medium 3; ▲, medium 4; □, medium 5; ■, medium 6; ▽, medium 7; ▼, medium 8; ★, medium 9; X, medium 10. The lines were calculated by the method of least squares.



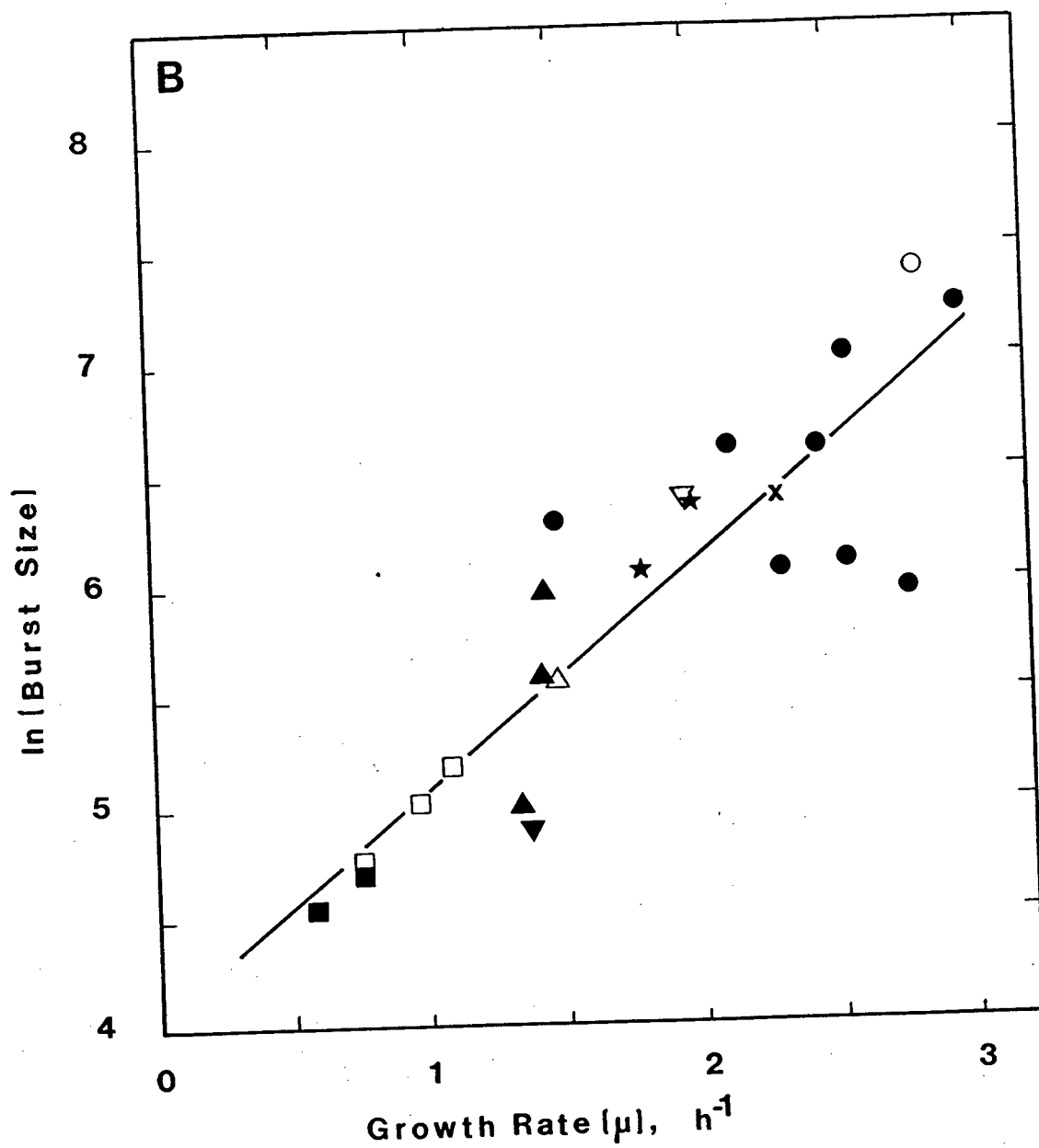


TABLE II

Exponential rate constants for total RNA per cell,
percent active RNA polymerase, and SP82 burst size

| Parameter | Exponential Rate Constant ^a |
|--|--|
| Total RNA per Cell ^b | 0.723 ± 0.108 |
| Percent Active RNA polymerase ^b | 0.943 ± 0.166 |
| SP82 Burst Size | 1.030 ± 0.250 |

^aThe slope of the line when the natural logarithm of the parameter is plotted against growth rate. Also given is the 95% confidence limits for the slopes of the lines (calculated by the method of Larsen and Marx, 1981).

^bTaken from Leduc et al. (1982).

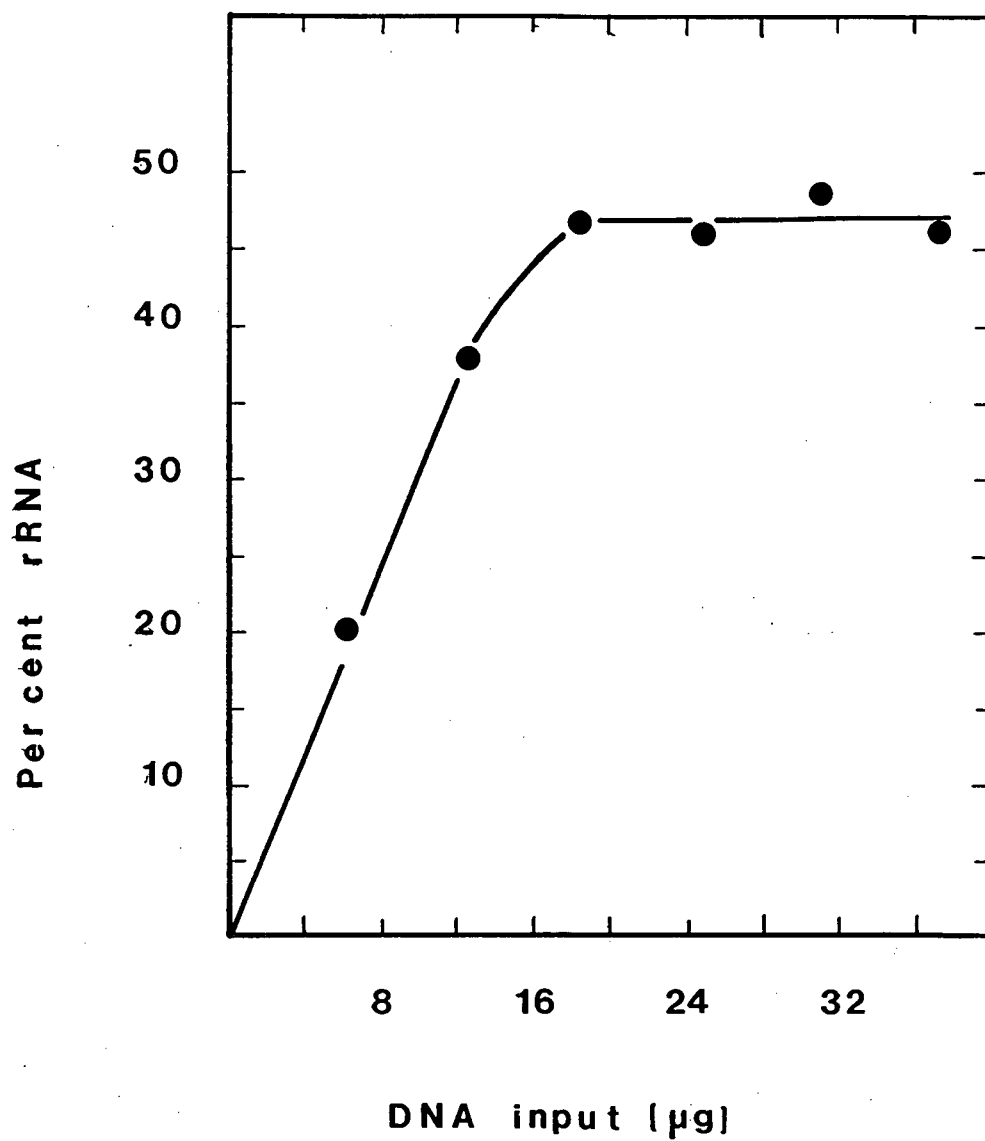
for the enteric bacteria (Maaloe and Kjeldgaard, 1966; Gausing, 1977; Quann, et al., 1980; Bremer and Dennis, 1987). The exponential rate constant for burst size against growth rate (the slope of the line in Figure 3b) and the exponential rate constants for total RNA per cell and percent active RNA polymerase against growth rate are shown in Table II (the latter two values were obtained from Leduc, et al., 1982). The difference in the constants for SP82 burst size and percent active RNA polymerase is not statistically significant, but the exponential rate constant for total RNA per cell is significantly different from the constant of the other two parameters. This result suggested that phage burst size might be dependent on active RNA polymerase and indicated that the HMU phage might be a suitable probe for RNA polymerase availability.

B. rRNA synthesis in an uninfected culture

1. Analysis of rRNA synthesis in Bacillus during steady state growth. Plasmid pVG-1 was used as a DNA probe for DNA-RNA hybridization to determine the percent rRNA synthesis. Cells were pulse labeled for 1 min with [³H]-uridine and a constant amount of the purified RNA was hybridized to increasing levels of pVG-1. The efficiency of the reaction was measured by hybridization of increasing concentrations of purified rRNA to 2 ug of rDNA and was characteristically high (88%). Binding to either blank filters or to filters carrying pBR322 DNA was negligible.

Results from a hybridization experiment are shown in Figure 4. RNA was labeled in cells grown in a DMT + succinate medium ($\mu=1.16h^{-1}$) and hybridized for 14 h to increasing amounts of pVG-1 DNA (achieved by including more DNA filters in the reaction vessel). Figure 4 shows a

Figure 4. Hybridization of pulse labeled RNA to cloned ribosomal DNA. Cells were grown in DMT + 0.25% succinate at a growth rate $\mu=1.16 \text{ h}^{-1}$. When culture density reached $5.7 \times 10^7 \text{ cells mL}^{-1}$. Twenty millilitres of cells were labeled with 200 uCi $[^3\text{H}]$ -uridine for 1 min. The specific activity of the purified RNA was 18,300 cpm μg^{-1} RNA. The hybridization reaction was for 14 h. Each vial contained 3.1 μg (57,000 cpm) of RNA. The percent rRNA synthesis (46.8% in this experiment) was calculated from the cpm of RNA bound at the plateau as described in Materials and Methods.



saturation curve with increasing input DNA which reaches a maximum at 18 ug with no additional increase up to 37 ug. This saturation level was used to calculate the percent rRNA levels. At least two factors might lead to an error in the estimation of this number. First, the distribution of labeled uracil into other pyrimidine nucleoside triphosphates was not taken into account. Second, although the curve appears to be saturating, it could be lower than true equilibrium. However, both these errors will be systematic, and will not affect comparisons between growth conditions. Although no estimation of the magnitude of these errors has been made, it is unlikely to be greater than $\pm 10\%$ of the final value.

To determine the range for the percent rRNA synthesis in Bacillus growing at different rates and to define conditions suitable for the shift-up experiments, cultures were grown in media yielding different growth rates. At a density of 1×10^8 cells/mL, the cells were pulse labeled with [^3H]-uridine for 1 min and the percent rRNA synthesis was determined. A steady increase in the percent of pulse labeled RNA which is ribosomal was seen over the growth rate range 0.68 h^{-1} to 1.88 h^{-1} (Figure 5). The rRNA synthesis varied between 43% and 63% at the extremes with an increase of 12.5%/u assuming the observed relationship was, as indicated, linear. The two sigma confidence of the relationship shown in Figure 5 was 0.91; shown are hybridization results obtained using the same RNA sample (growth rates 1.88 and 1.16), which can be used to assess the accuracy of the data presented.

Figure 5. Rate of ribosomal RNA synthesis as a function of growth rate. Cumulative results of nine hybridization experiments using pulse labeled RNA from seven different steady state cultures. The per cent rRNA synthesis calculated from the saturation level of radioactivity bound to pVG-1 is shown as a function of growth rate, μ .

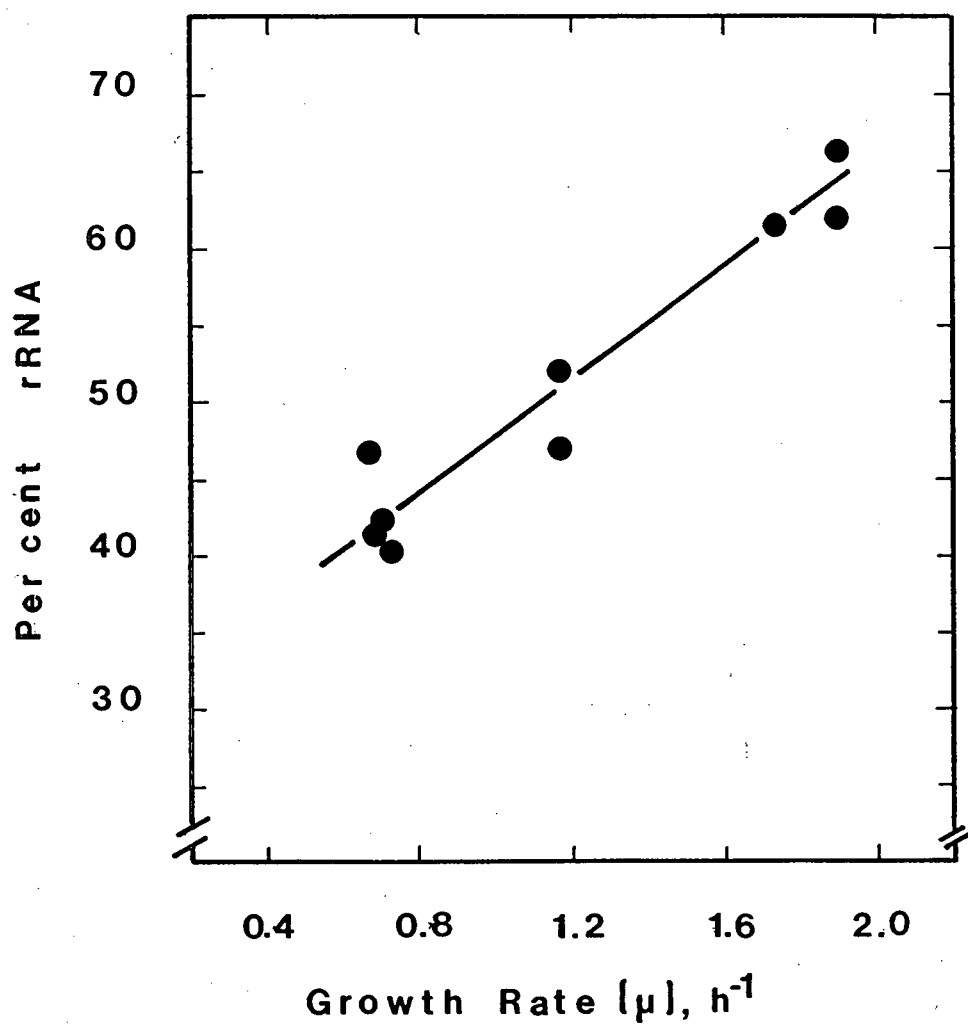
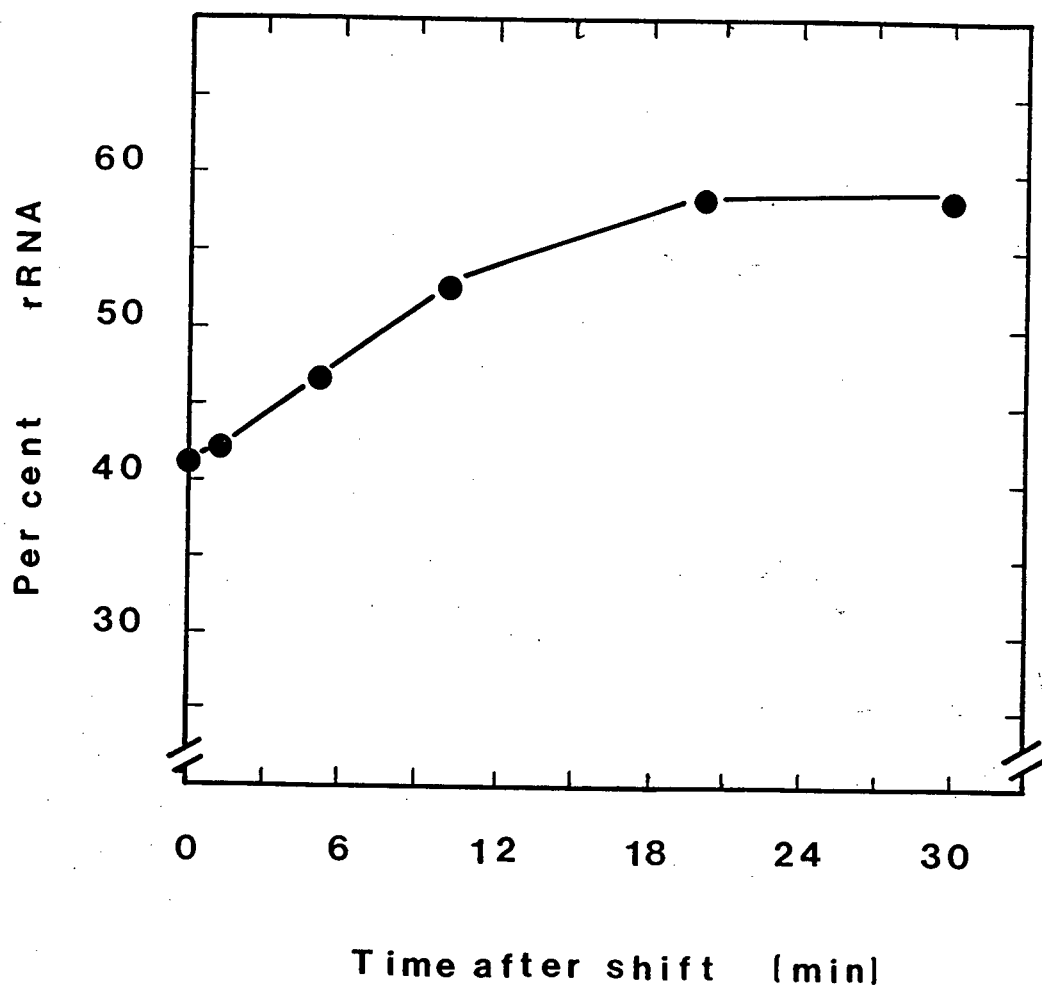


Figure 6. The kinetics of ribosomal RNA synthesis following a nutritional shift-up. Cells were grown in DMT + 0.25% acetate + 0.2% casamino acids at a growth rate $\mu=0.68 \text{ h}^{-1}$. When culture density reached $6.2 \times 10^7 \text{ cells mL}^{-1}$ the culture was shifted to (final concentration) 0.1% glucose, 0.5% casamino acids, 6 mg/mL adenosine, and vitamins. At the times indicated 10 mL of culture were removed and labeled with 200 μCi $[\text{H}^3]$ -uridine for 1 min. The per cent rRNA synthesis was calculated from input radioactivity bound to pVG-1 at DNA saturation.



2. Kinetics of rRNA synthesis following a nutritional shift-up.

The change in kinetics of rRNA synthesis during a nutritional shift-up is shown in Figure 6. Cells were grown in DMT-acetate medium to a density of 5×10^7 cells/mL. A prewarmed concentrated solution of glucose + casamino acids was added to a final concentration of 0.1% glucose and 0.5% casamino acids, 0.6 mg/mL adenosine and 1x vitamins (Rodin, 1972) with a volume increase of 10%. After addition of the nutrients 2mL samples were removed and pulse labeled for 1 min. RNA was extracted and hybridized to determine the percent rRNA synthesis. The percent rRNA synthesis appeared to remain constant for 1 min and then increased rapidly up to 18-20 min after the shift when it became stable again. The data showed that labeling times between 5 and 10 min after nutritional shift should allow monitoring of the shift in rRNA synthesis.

C. rRNA synthesis in a phage infected culture

To examine the effect of SP01 RNA polymerase modification on rRNA synthesis, a culture of B. subtilis was grown in DMT + acetate medium. When the density reached 6×10^7 cells/mL, the culture was split into control and experimental aliquots. The experimental culture was then infected with SP01am34 carrying an amber mutation in one of the two proteins involved in the second polymerase modification (see Introduction to this section). After 10 min of infection, one-half of both the phage infected and uninfected cultures was subjected to nutritional shift-up by the addition of concentrated medium. After 8 min of further incubation to allow expression of increased rRNA synthesis, samples of all 4 cultures were pulse labeled and the isolated RNA analyzed by hybridization. Examples of the hybridization data are shown in Figure 7 and Table III.

Figure 7. The effect of SP01am34 infection on the synthesis of ribosomal RNA in response to a nutritional shift-up. Cells were grown in DMT + 0.5% acetate + 0.2% casamino acids at a growth rate $\mu=0.68 \text{ h}^{-1}$. When culture density reached $6 \times 10^7 \text{ cells mL}^{-1}$ an aliquot was infected at an $\text{moi}=25$ (panel B). Ten minutes after infection both infected and uninfected cultures were shifted to M medium (final concentration). These cultures were labeled 8 min after the shift-up (18 min post infection), and the per cent rRNA synthesis was measured as described in Figure 4 and Materials and Methods. Hybridization reactions were for 21 h. Symbols are: unshifted uninfected (open triangles); shifted uninfected (closed triangles); unshifted infected (open circles); shifted infected (closed circles).

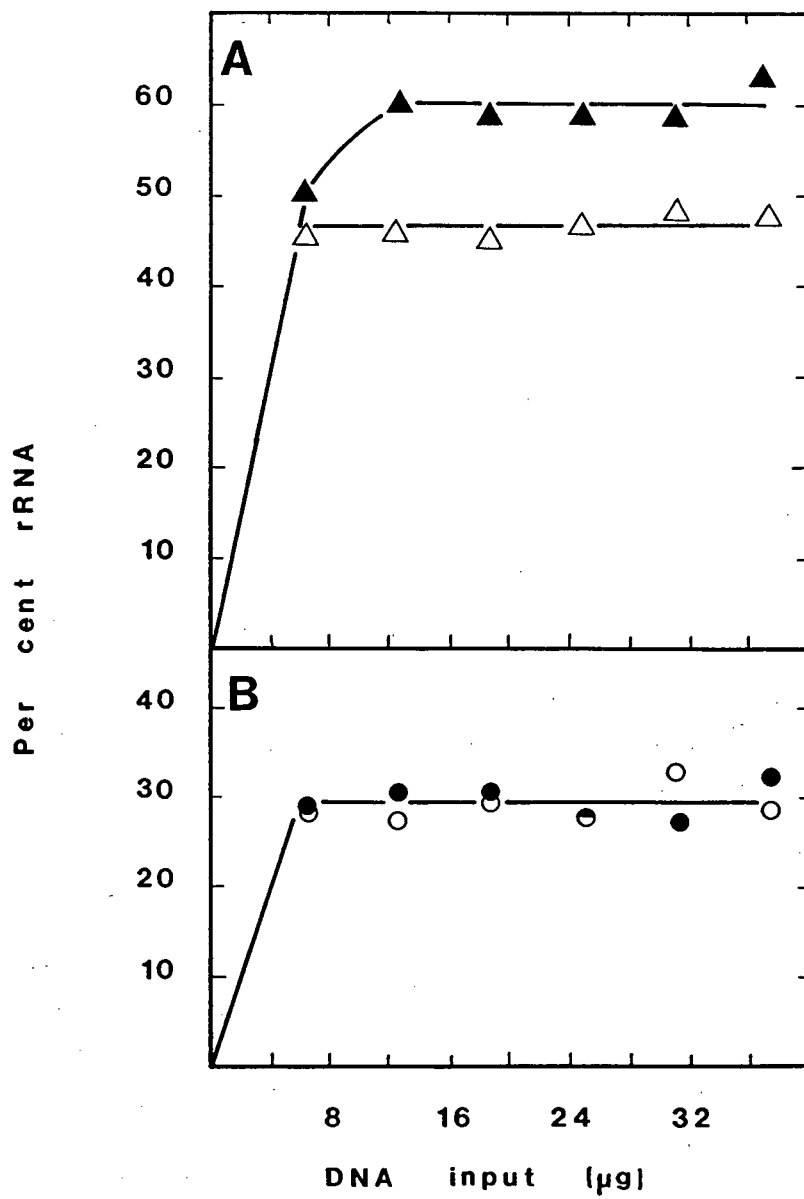


TABLE III

Summary of Shift Up Experiments

| <u>labeling Conditions</u> | | Percent rRNA synthesis ^a | |
|----------------------------|------------|--|----------------|
| | | <u>Unshifted</u> | <u>Shifted</u> |
| label 5 min after shift | uninfected | 41.4 \pm 1.5 | 50.2 \pm 1.4 |
| | infected | 38.5 \pm 2.9 | 39.2 \pm 1.4 |
| label 8 min after shift | uninfected | 47.4 \pm 1.1 | 59.3 \pm 1.6 |
| | infected | 30.9 \pm 1.1 | 31.0 \pm 1.6 |
| label 8 min after shift | uninfected | 31.4 \pm 2.0 | 46.1 \pm 2.2 |
| | infected | 36.1 \pm 1.8 | 38.4 \pm 1.4 |

^a Calculated from the input radioactivity (³H-uridine RNA) from cultures (either infected, or uninfected and with and without nutritional shift-up) binding to rDNA.

In panel A of Figure 7, hybridization data from the uninfected control cells are illustrated. Addition of the nutrients stimulated the percent rRNA synthesis from 47% to 58% within 8 min after the addition of nutrients. The extent of this change is similar to that seen in Figure 6 where 8 min after the shift-up the percent rRNA synthesis increased from 40 to 52%. Panel B, Figure 7, shows the hybridization data using RNA isolated from the infected cells. The pulse labeling of the infected cells in Figure 7 took place a total of 18 min after phage infection allowing ample time for modification of the host polymerase (Fox, 1976).

The data in Figure 7 show that the percent rRNA synthesis was only marginally changed by SP01am34 infection. In Figure 7 the unshifted rRNA synthesis rate was somewhat lower after infection than before, however in other trials (Table III), addition of the phage had no effect on rRNA synthesis. The most striking point illustrated in Figure 7 (panel B) is that addition of the phage completely blocked the increase in rRNA synthesis stimulated by addition of rich media. As shown in Table III, slight variations in timing of shift or pulse labeling had no effect on the result that phage infection blocked the shift-up response. In all cases phage infected cells carried on rRNA synthesis at roughly preinfection rates but could not undergo shift-up in rRNA production. These data suggested that there might be an RNA polymerase specific for rRNA synthesis and that that form of polymerase was "immune" from gp28 modification. These data also indicated that the hypothesis that phage burst size might be dependent on active RNA polymerase cannot be correct, since the fraction of polymerase synthesizing rRNA (and hence "immune" from gp28 modification) would increase with growth rate.

D. Summary of in vivo results

The results presented in this section showed the following:

1. SP01 infection did not appreciably affect the rate of transport of amino acids and glucose into the infected cell; however, the rates of nucleosides and base transport were reduced to 40-60% of the control.

2. SP82 could productively infect *Bacillus* cultures growing in poor medium. Burst size increased logarithmically with growth rate.

3. The percent of total RNA that is rRNA synthesized at any one instant during steady state growth increased 12.5%/u.

4. In shift-up experiments the percent rRNA synthesized appeared to remain constant 1 min after the addition of nutrients, and then increased rapidly for 18-20 min.

5. Ribosomal RNA continued to be synthesized during SP01am34 infection at preinfection levels. The percent rRNA synthesized, however, did not increase when phage infected cultures were provided rich medium effecting a nutritional shift-up.

IV. IN VITRO RESULTS

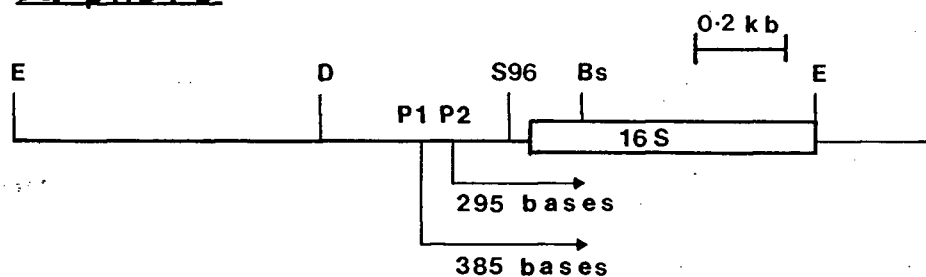
A. Templates used for in vitro transcription studies

Figure 8 shows the organization of the rRNA rrnB operon of B. subtilis and the structure of subsequent plasmid constructions. The plasmid pHD1.8 (Deneer and Spiegelman, 1987) served as the primary template in this study. For in vitro transcriptions pHD1.8 was linearized at the unique BstEII site, and the runoff transcripts which originated at P1 (384 bases) and P2 (294 bases) were quantified as described in Materials and Methods. Also shown in Figure 8 are three subcloned constructions of the promoter region. Plasmid pKK427B contains both promoters of the rrnB operon, but has only 200 bases 5' of the P1 promoter and 37 bases 3' of the P2 promoter (Deneer and Spiegelman, 1987). In vitro transcriptions using pKK427B linearized with BamHI produced a 200 base P1 transcript and a 110 base P2 transcript. The tandem promoters were separated as summarized in Figure 8 (H. Deneer, Ph.D. Thesis, University of British Columbia, 1986). For in vitro transcription both plasmids were linearized with BamHI. The separated P1 construct, pTLXT-205, produced a 65 base runoff transcript, while the separated P2 construct, pTLXT-220, produced a 110 base transcript.

Figure 9 shows the structure of the two plasmids containing non-rRNA promoters which served as controls in these studies. The plasmid p328-5 is a pBR322 derivative which contains the A2 promoter from Bacillus phage ϕ 29 (Dobinson and Spiegelman, 1985). In vitro transcription using p328-5 which had been treated with EcoRI produced a 236 base runoff transcript. The plasmid p679 is a pEMBL8 derivative

Figure 8. Structure of plasmids containing the promoter regions of the rrnB operon. A. The plasmid pHD1.8 was constructed by inserting the 1900 bp EcoRI fragment of pGS227 which contains the B. subtilis rrnB tandem promoter (Stewart and Bott 1983) into the EcoRI site of pKM-1 (Deneer and Spiegelman, 1987). B. Plasmid pKK427 contains a 512 bp DdeI fragment from the 1900 bp insert of pHD1.8 which was isolated and then digested with Sau96I and cloned into the SmaI site of pKM-1 (Deneer, Ph. D. thesis). Heavy lines represent cloned, non-coding regions 5' to the structural gene. Open box represents the coding region of the 16S gene. Thin lines represent vector DNA. To separate the promoters, a 427 bp fragment from pKK427 was digested with Sau3A and HincII and a 205 bp fragment whose 3' terminus ended in the -35 region of the P2 promoter was inserted into the BamHI site of pTLXT-11 (Deneer, Ph. D. thesis). The resulting plasmid, pTLXT-205, contains only the P1 promoter intact. To yield a plasmid with only P2, pKK427 was cut with EcoRI, treated with Bal31 exonuclease. One of the deletions (pTLXT-220) was sequenced and the 5' end was found to lie between the -10 and -35 regions of P1 (indicated by x), inactivating the promoter (Deneer, Ph. D. thesis). Restriction sites are designated as follows: EcoRI (E), BamHI (B), BstEII (Bs), Sau3A (S3), Sau96I (S96), DdeI (D), and HincII (Hc). Restriction sites in parenthesis have been destroyed by cloning but are shown for reference. The lengths of the runoff transcripts produced in in vitro transcription reactions are indicated.

A. pHD1.8



B. Promoter region subclones

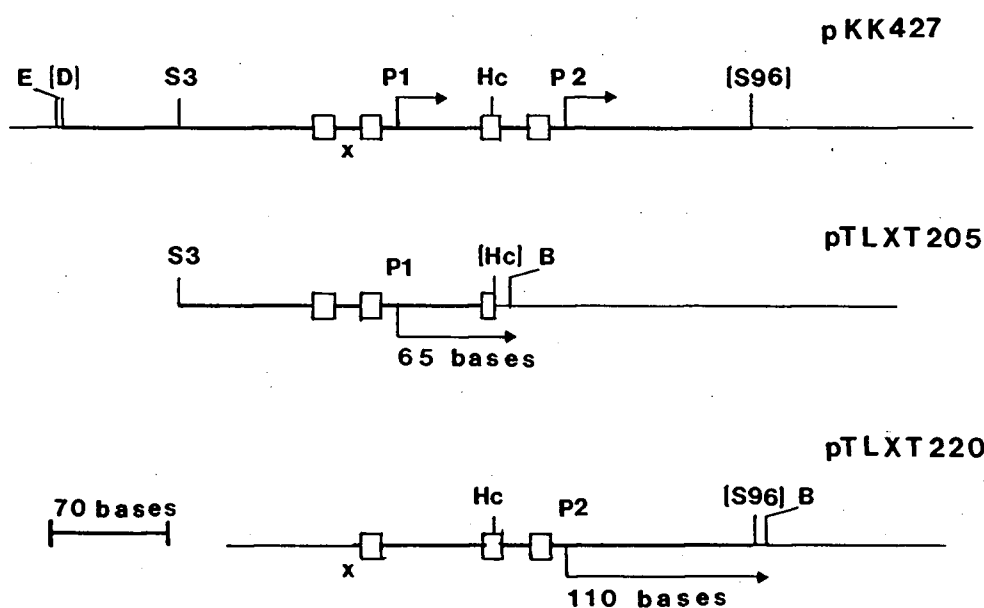
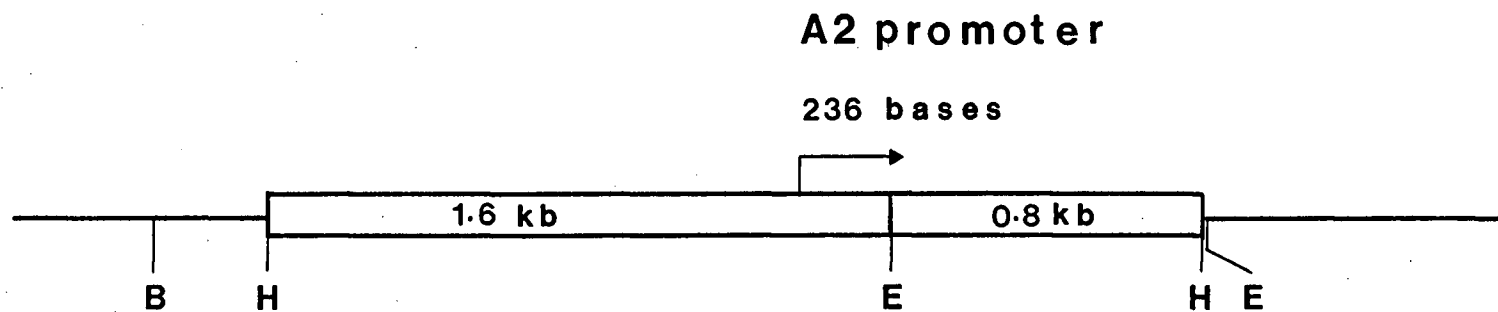
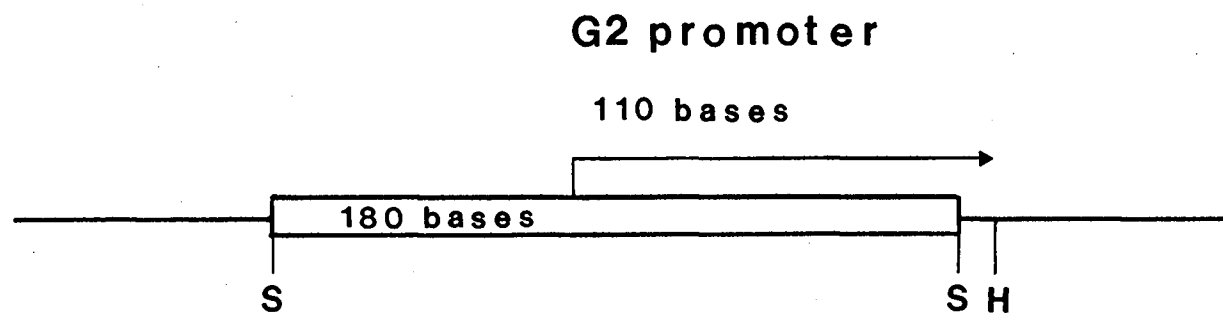


Figure 9. Structure of plasmids containing the promoter region of two ϕ 29 promoters. A. Plasmid p328-5 contains a 2.4 kb fragment from ϕ 29 DNA, delineated by HindIII restriction endonuclease sites, was isolated and cloned into pBR322 (Dobinson and Spiegelman, 1985). B. Plasmid p679 contains a HindIII to HinfI restriction endonuclease fragment from the right terminus of ϕ 29 genome, cloned into the SmaI site of pEMBL-8 (Dente *et al.*, 1985). The ϕ 29 fragment contains the G2 promoter (Garvey *et al.*, 1985; Dobinson and Spiegelman, 1987). Restriction sites are designated as follows: HindIII (H), EcoRI (E), BamHI (B), and SmaI (S). The lengths of the runoff transcripts produced in *in vitro* transcription reactions are indicated. Open boxes represents cloned ϕ 29 DNA, thin lines represent vector DNA.

A. p328-5



B. p679



which contains the G2 promoter of phage ϕ 29 (Dobinson and Spiegelman, 1987). A 110 base runoff transcript was produced when HindIII treated p679 was used as template in in vitro transcription reactions.

B. Isolation of rRNA specific RNA polymerase

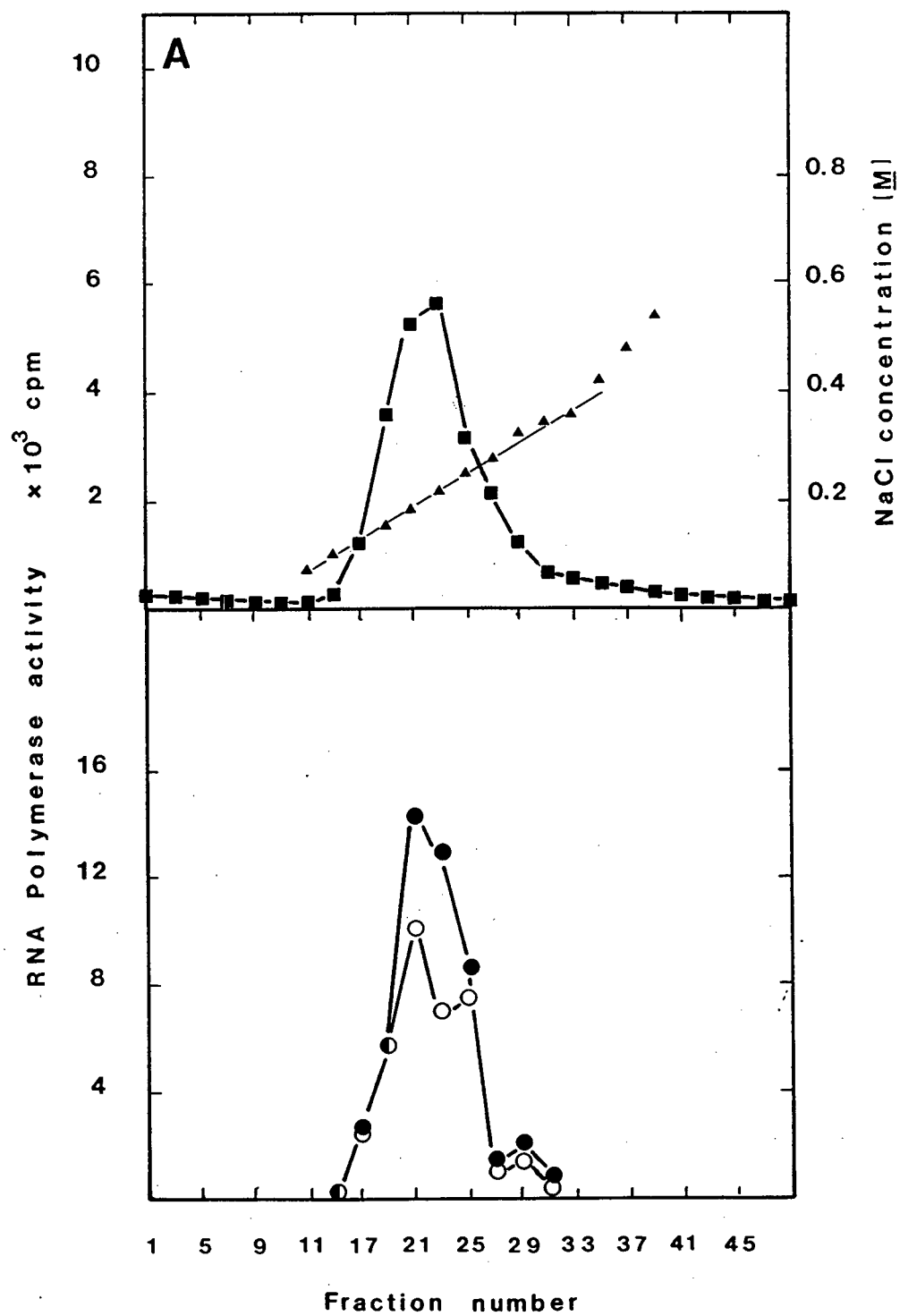
Although SP01 infected cultures could not carry out the nutritional shift-up response of an increased rate of rRNA synthesis, the observation that rRNA continued to be synthesized at preinfection levels (see above) suggested that there might be an rRNA specific RNA polymerase that was "immune" from phage modification by gp28 (Fujita, et al., 1971; Fox, 1976; Duffy and Geiduschek, 1975). Two approaches were taken toward the isolation of such an rRNA specific RNA polymerase. The basic assumption in the first approach was that an rRNA specific enzyme might have increased affinity for DNA containing the promoter region of an rRNA operon, and hence, might be isolated by chromatography on a specific DNA-cellulose and subsequent elution with a salt gradient. The assumption in the second approach was based on the observations of Travers, et al. (1980) that on zone sedimentation E. coli RNA polymerase exhibited functional heterogeneity with respect to template preference, regulation by ppGpp, and affinity for fMet-tRNA. The following are the results of preliminary experiments suggested by the above approaches. In both cases no significant differences were observed between RNA polymerase activity on the A2 promoter and activity on the rRNA tandem promoters.

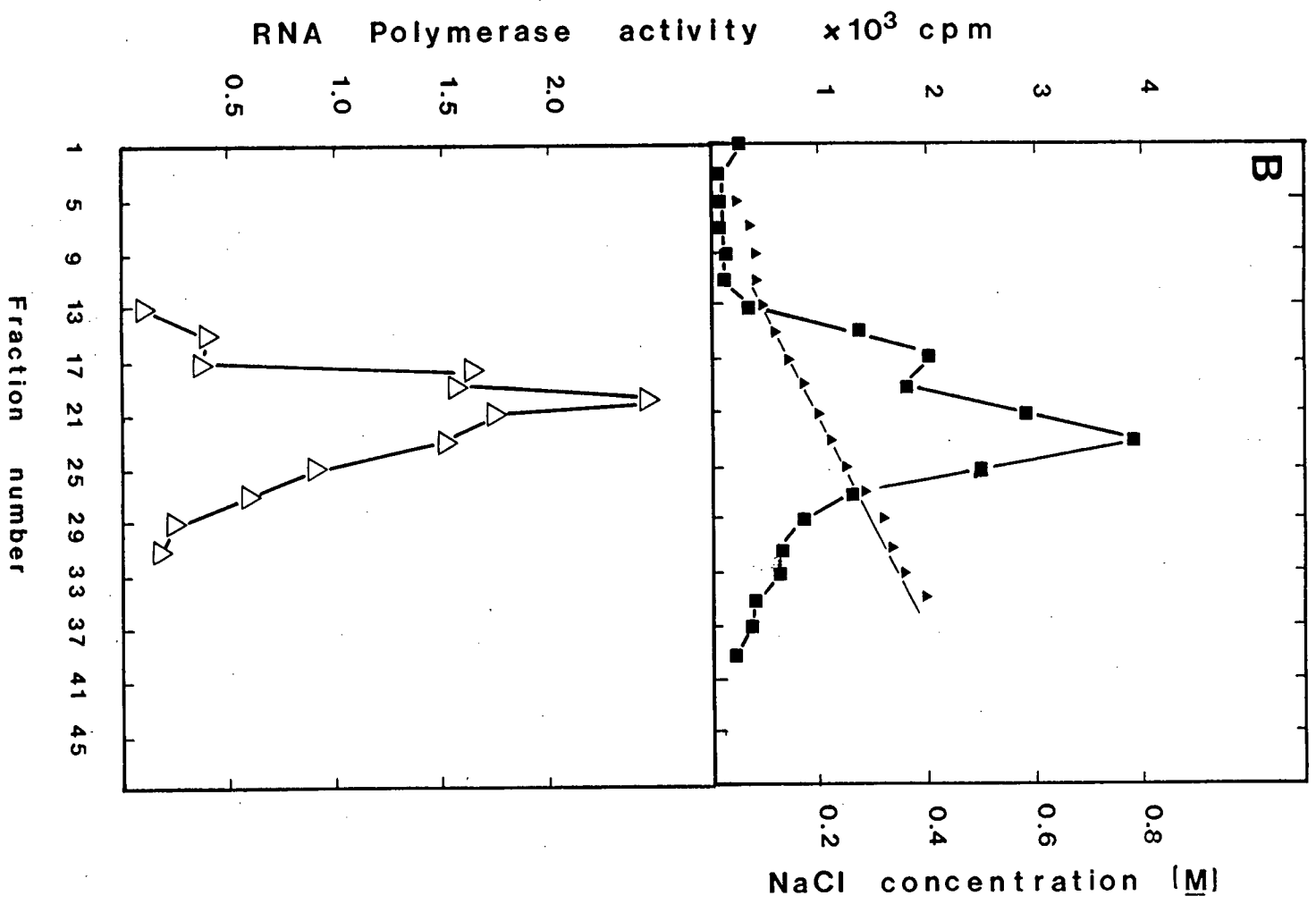
1. Affinity chromatography--For the affinity studies RNA polymerase was purified from B. subtilis 168 as described in the Materials and Methods except that sedimentation through the glycerol

gradient was omitted. One and one-half millilitres of the pooled 0.6 M NaCl fractions from the first heparin-sepharose chromatography were loaded on a 1.5cc PHD1.8 DNA-cellulose column and eluted with a 0.05-0.8 M or a 0.05-0.6 M NaCl gradient. Four drop fractions were collected and assayed for RNA polymerase activity as described previously (Dobinson and Spiegelman, 1985). Fractions containing peak activity were used in a standard transcription assay with PHD1.8 (rrnB tandem promoter, Figure 10a) or p328-5 (ϕ 29 A2 promoter, Figure 10b) as template to assess enzyme specificity. Figure 10 shows the elution profile of total RNA polymerase activity, the enzyme activity on the specific templates and the NaCl concentration for two different enzyme samples chromatographed on PHD1.8 DNA-cellulose. In both experiments the total activity peak occurred at a higher salt concentration than the specific activity peak. Total polymerase activity eluted as a broad peak with an estimated maximum eluting at 0.22 M NaCl, while maximum polymerase activity assayed on the specific templates eluted at 0.18 M NaCl for both the tandem rRNA promoters (Figure 10a) and the A2 promoter (Figure 10b). The assay for total activity measures the RNA synthesized by both core (α_2 , β , β') and holoenzyme (core + σ^{43}), whereas the assay for specific transcripts measures the RNA synthesized by only holoenzyme. The different elution patterns of total activity and specific activity may reflect the different affinities of core and holoenzyme for sites on the DNA cellulose.

2. Sedimentation--For the sedimentation studies RNA polymerase from B. subtilis 168 (Iowa Grain Processing) which had been purified through the DNA cellulose affinity chromatography step (Dobinson and Spiegelman, 1985) was used. The fractions which showed the peak polym-

Figure 10. Elution profile of RNA polymerase activity from pHD1.8 DNA-cellulose column. One and a half millilitres of heparin-sepharose purified RNA polymerase were loaded onto 1.5cc pHD1.8 DNA-cellulose column. A. Column was eluted with a 0.05-0.8 M NaCl gradient. B. Column was eluted with a 0.05-0.6 M NaCl gradient. Total RNA polymerase activity (closed squares) and NaCl concentration (closed triangles) are shown in the upper part of each panel. Enzyme activity at specific promoters was determined using the standard transcription assay described in Methods and is shown in the lower part of each panel: P1 (open circles), P2 (closed circles), and A2 (open triangles).

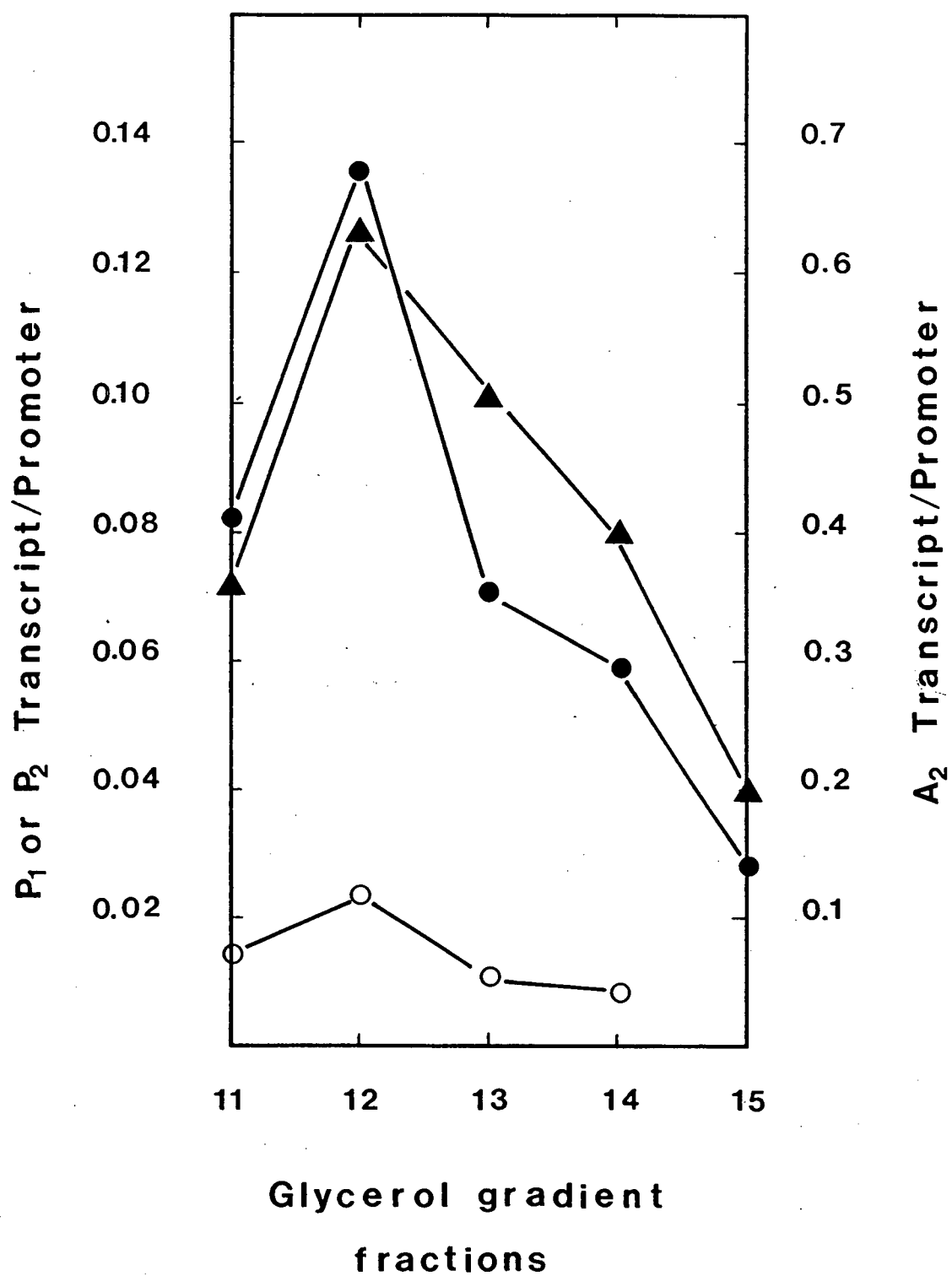




erase activity were concentrated, loaded on a 15-30% glycerol gradient and sedimented as described in Methods. The fractions which showed the peak polymerase activity were assayed for protein concentration and used in single round transcription assays at 20 nM RNA polymerase with either the rRNA tandem promoter construct or the ϕ 29 A2 promoter construct as template (Figure 11). The tandem rRNA promoters were found to be highly sensitive to the nucleotide composition of the initiation mix (see below). The results presented in Figure 11 for the rRNA promoters are from separate assays in which the pHD1.8 template was incubated with GTP, ATP, and UTP (GAU, P1 data) or ATP, GTP, and CTP (AGC, P2 data) and enzyme before heparin and the fourth nucleotide were added. The RNA polymerase was more active at P1 when GAU was in the initiation mix than when AGC was present, and conversely, the enzyme was more active at P2 when AGC was in the initiation mix than when the mix contained GAU (see below). Despite the variation in the absolute level of RNA polymerase activity at each promoter, the peak of activity occurred in fraction 12 for all promoters suggesting that an rRNA specific polymerase was not isolated by this method.

These preliminary experiments failed to isolate an RNA polymerase which would preferentially transcribe rRNA genes. Routinely purified enzyme fractions produced transcripts at a reproducible level from rRNA and non-rRNA promoters in the single round transcription assay. These data suggested that regulation of rRNA synthesis might take place at RNA polymerase-promoter interactions. The focus of the in vitro study of rRNA synthesis, therefore, widened to include the nature of the interaction between polymerase and the rRNA promoters.

Figure 11. Specific RNA polymerase activity in glycerol gradient fractions. Total RNA polymerase activity occurred in gradient fractions 11-15 and reached maximum in fraction 14. Enzyme activity at specific promoters was determined using the single round transcription assay described in Methods. The incubation reaction for transcription from the P1 promoter (open circles) contained 400 μ M GTP, CTP and 10 μ M ATP; UTP was added to 400 μ M with heparin. The incubation reaction for transcription from the P2 promoter (closed circles) contained 400 μ M GTP, UTP, and 10 μ M ATP; CTP was added to 400 μ M with heparin. The incubation reaction for transcription from the A2 promoter (closed triangles) contained 400 μ M ATP and GTP; CTP (400 μ M) and UTP (10 μ M) were added to with heparin. Heparin mix was added 1 min after the enzyme and elongation was permitted for 10 min. Transcription products were separated by electrophoresis and quantified as described in Methods. The final enzyme concentration from all fractions was 20 nM.

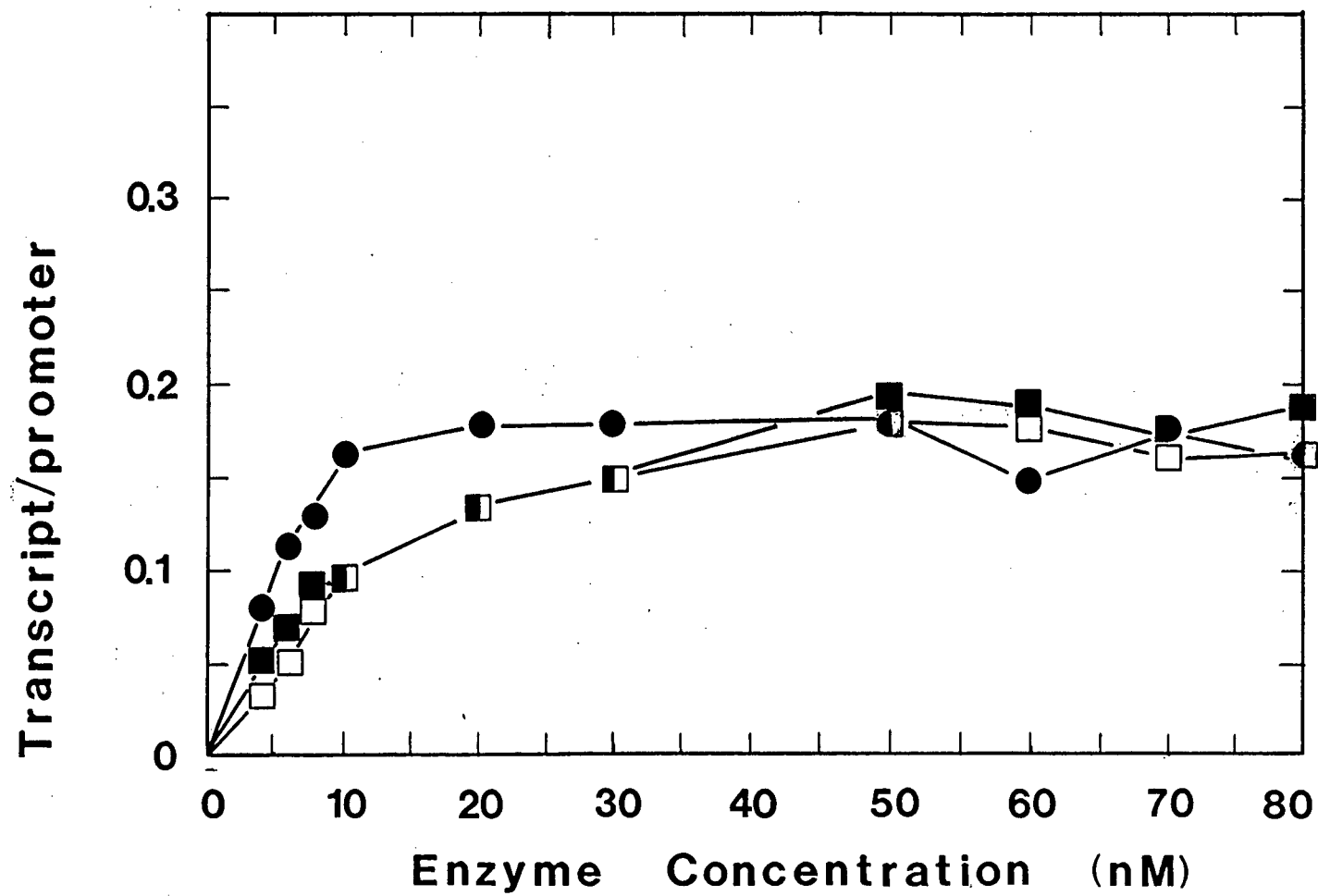


C. Effect of assay parameters on in vitro transcription of the tandem rRNA promoters

To begin the investigation of transcription initiation at the individual rRNA promoters, the effect of different kinetic parameters on RNA polymerase activity on the tandem promoter construct was studied. RNA polymerase activity at the rRNA promoters in the tandem construct exhibited some unexpected characteristics when compared to activity at the previously described bacteriophage $\phi 29$ promoters (Dobinson and Spiegelman, 1985 & 1987). In the following experiments RNA polymerase activity was measured by following the synthesis of transcripts produced when mixtures of polymerase and promoter were challenged with heparin. The experiments thus measure the formation of complexes which can initiate in the presence of heparin and are thus called heparin resistant complexes.

Figure 12 compares the formation of heparin resistant complexes at the $\phi 29$ A2 promoter and at the tandem rRNA promoters as a function of enzyme concentration. In this experiment the reaction mixture with the A2 template (at 2 nM) included ATP and GTP (AG), while the reaction mixture with the tandem rRNA promoter template (at 2.6 nM) included GTP, UTP, and CTP (GUC). As the enzyme concentration increased, the level of heparin resistant complexes reached a plateau indicating that either the DNA was saturated with enzyme or a steady state had been reached. The rRNA promoters required a higher enzyme concentration to reach their plateau level (30-50 nM) than did the phage promoter (10nM). This result was rather surprising in light of the strength of the rRNA promoters in vivo. Figure 12 also shows that even at the highest enzyme concen-

Figure 12. The effect of RNA polymerase concentration on transcription initiation from A2 (closed circles), P1 (open squares), and P2 (closed squares) promoters. RNA polymerase at the concentrations indicated was incubated with linearized templates of either the A2 promoter (2 nM) in the presence of 400 μ M ATP and GTP, or the rRNA tandem promoter (2.6 nM) in the presence of 400 μ M GTP, UTP, and CTP for 10 minutes, at which time heparin (5 μ g/mL, final concentration) and the remaining nucleotides were added and the elongation reaction allowed to proceed for a further 10 minutes. Transcription products were separated by electrophoresis and quantified as described in Methods.



trations the amount of heparin used was sufficient to stop multiple rounds of transcription initiation. No more than 0.3 transcripts per promoter were produced at either the A2 template or the tandem rRNA promoter template at the higher enzyme inputs. Dobinson and Spiegelman (1987) reported a comparable level of polymerase activity at the A2 promoter.

A second unexpected characteristic of the rrnB tandem promoters is illustrated by the data in Figure 13. The rate of heparin resistant complex formation at the ϕ 29 A2 and G2 promoters and the tandem rRNA promoters was measured in the presence of AG, for the former, or GUC, for the latter. Complexes formed very rapidly on both phage promoters and reached maximum levels in approximately one minute. Similar kinetics were reported by Dobinson and Spiegelman (1985, 1987; see below). In contrast, the rRNA tandem promoters were relatively inefficient substrates for complex formation as it took 4 min to reach the maximum level of complexes. In all cases the level of complexes remained constant for up to 15 min. Furthermore the slow rate of complex formation was observed with different enzyme batches and appeared to be a characteristic of the holoenzyme.

A third unexpected characteristic of transcription initiation at the rRNA promoters was the apparent sensitivity of the reaction to the presence of specific nucleotides in the initiation mixture. Heparin resistant complexes formed at both P1 and P2 of the tandem promoter construct when the initiation mix included GUC (see above). When the separated promoter constructs served as templates a different result was seen. Figure 14a shows an autoradiogram of an enzyme concentration

Figure 13. The effect of initiation time on transcription initiation from A2 (closed circles), G2 (open circles), P1 (open squares), or P2 (closed squares) promoters. RNA polymerase (20 nM) was incubated with linearized templates of either the A2 promoter (2 nM), G2 promoter (2.3 nM), or the rRNA tandem promoter (2.6 nM) for the times indicated. The A2 and G2 templates incubation mixes contained 400 uM ATP and GTP, and the rRNA tandem promoter template incubation mix contained 400 uM GTP, UTP and CTP. After the initiation period 38 uL aliquots were removed and added to 2 uL heparin (5 ug/mL, final concentration) and the remaining nucleotides. Transcription products were separated by electrophoresis and quantified as described in Methods.

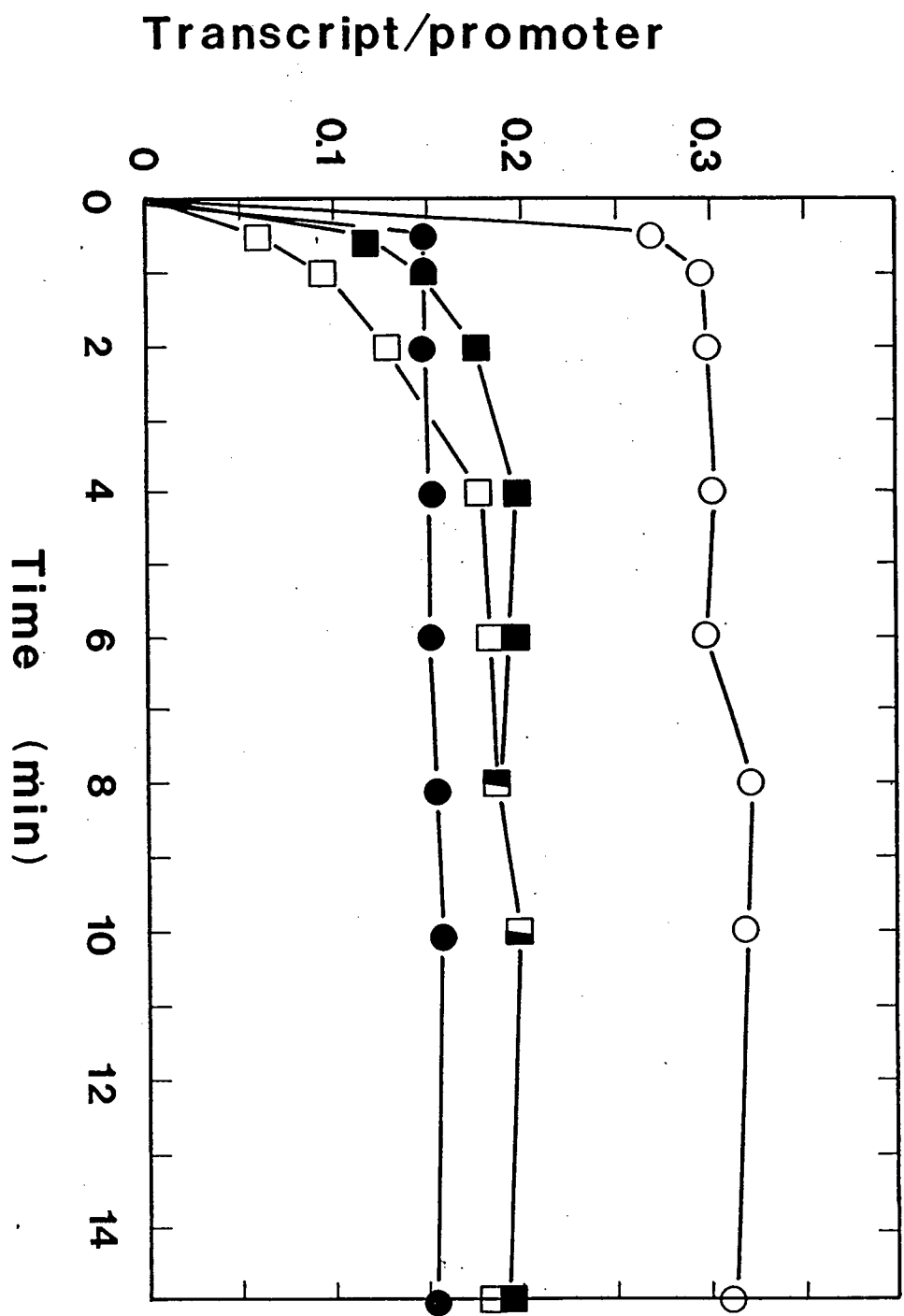
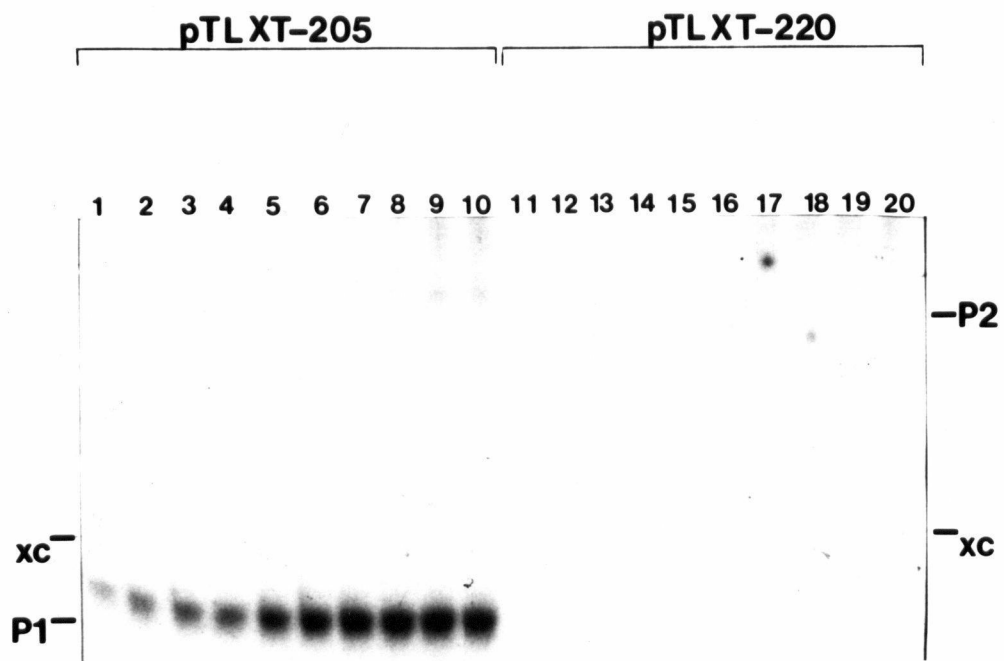
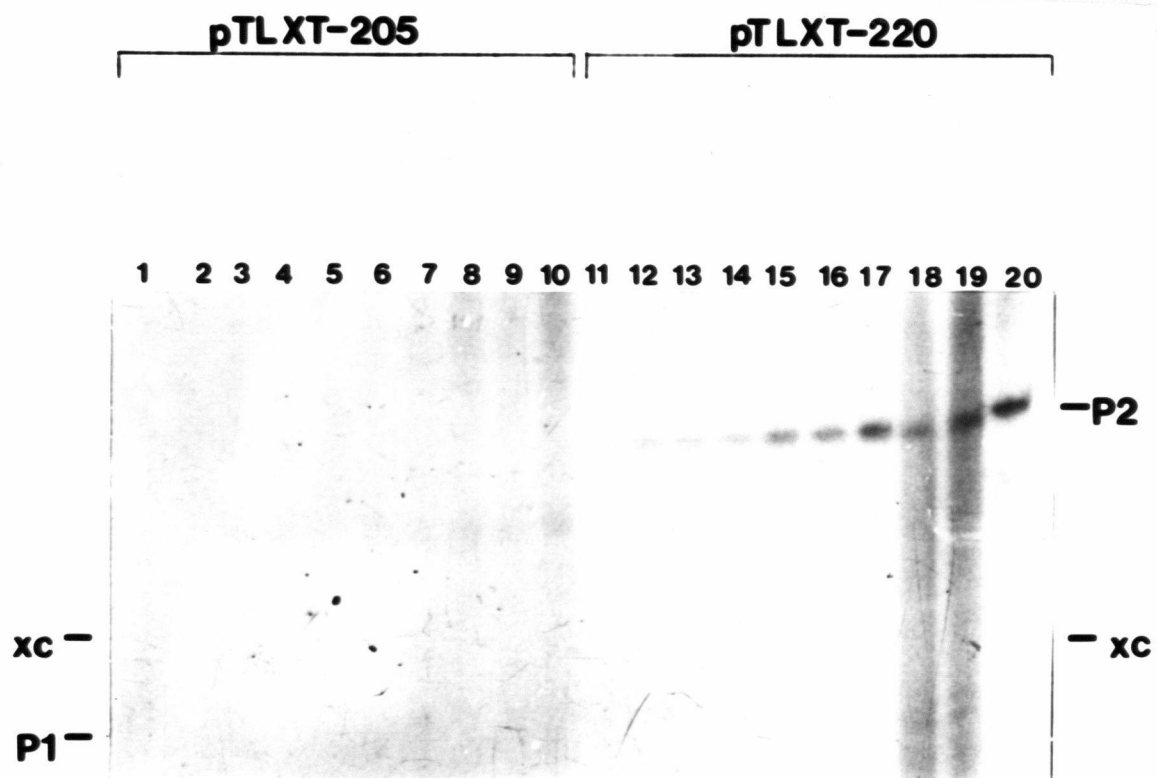


Figure 14. The effect of the nucleotide composition of the initiation mix on transcription initiation from the separated ribosomal promoters. Plasmid pTLXT-205 containing only the P1 promoter (lanes 1-10) or plasmid pTLXT-220 containing only the P2 promoter (lanes 11-20) were incubated in a solution containing 400 μ M GTP, UTP, CTP (panel A) or 400 μ M ATP, GTP, CTP (panel B) with RNA polymerase at the following concentrations for 10 min: 4 nM, lanes 1 and 11; 6 nM, lanes 2 and 12; 8 nM, lanes 3 and 13; 10 nM, lanes 4 and 14; 20 nM, lanes 5 and 15; 30 nM, lanes 6 and 16; 50 nM, lanes 7 and 17; 60 nM, lanes 8 and 18; 70 nM, lanes 9 and 19; or 80 nM, lanes 10 and 20. Heparin and the remaining nucleotide ($[\alpha\text{-}^{32}\text{P}]$ labeled) were added to 5 μ g/mL and 10 μ M, respectively, and incubated for a further 10 min. Transcription products were separated by electrophoresis and the acrylamide gel subjected to autoradiography.

A. GUC initiation



B. AGC initiation



experiment in which the plasmids containing the separated P1 promoter, pTLXT205, (lanes 1-10), or the separated P2 promoter, pTLXT220, (lanes 11-20), served as templates and the initiation mix contained GUC. RNA polymerase formed heparin-resistant complexes only at the P1 promoter, but not at the P2 promoter. On the other hand, when an enzyme concentration experiment was performed using the same templates but incubated with AGC, the polymerase formed heparin-resistant complexes at the P2 promoter (Figure 14b, lanes 11-20), but not at the P1 promoter (Figure 14b, lanes 1-10).

D. Transcription from P1 affects transcription from P2

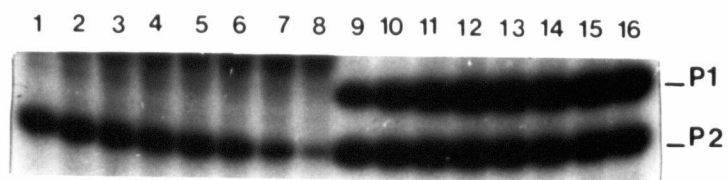
Changes in the initiation mix were found to influence the formation of heparin resistant complexes at a particular promoter and the stability of the enzyme in heparin-resistant complexes. Both formation and stability effects are illustrated in Figure 15. Figure 15a shows an autoradiograph of a polyacrylamide gel of transcripts produced in an initiation time course experiment employing 20 nM RNA polymerase. In the reactions shown in lanes 1-8 the initiation reaction contained nucleotides AGC. Transcripts were produced from P2 only. In the reactions shown in lanes 9-16 the initiation reaction contained GUC and transcripts were initiated at both P1 and P2.

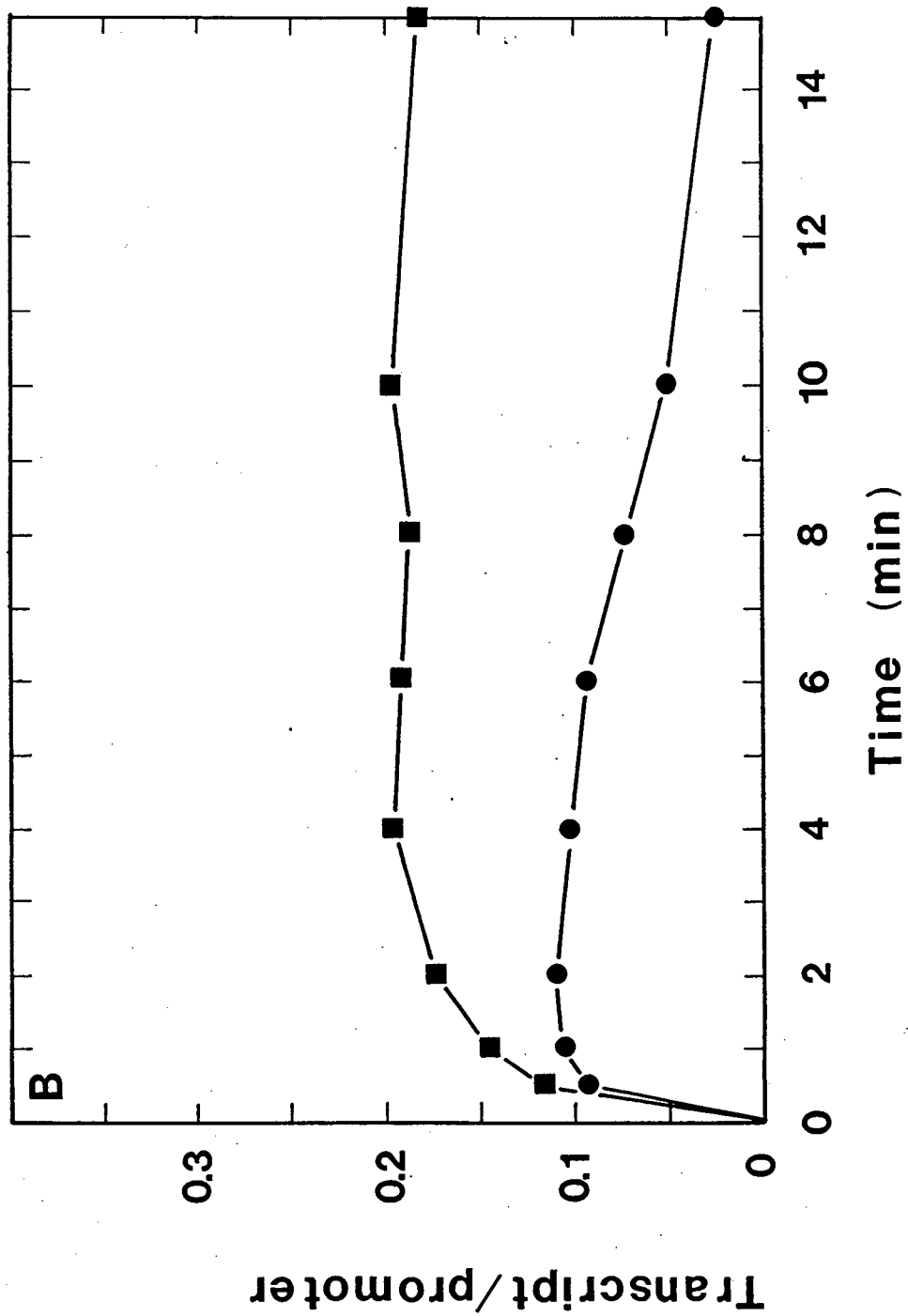
The effect on the stability of the heparin-resistant complexes formed at P2 can be more readily seen in Figure 15b, where the level of transcripts from P2 has been plotted as a function of incubation time. When heparin-resistant complexes could be formed at P1 (GUC initiation), heparin resistant complexes at P2 were stable to incubation time, since

Figure 15. The effect of initiation nucleotides on transcription initiation from P2. A. Autoradiogram of polyacrylamide gel of transcription products. RNA polymerase (20 nM) was incubated with the rRNA tandem promoter template (2.6 nM) in the presence of 400 uM ATP, GTP and CTP (lanes 1-8) or 400 uM GTP, UTP, and CTP (lanes 9-16). Initiation reactions were for 0.5 min (lanes 1 and 9), 1 min (lanes 2 and 10), 2 min (lanes 3 and 11), 4 min (lanes 4 and 12), 6 min (lanes 5 and 13), 8 min (lanes 6 and 14), 10 min (lanes 7 and 15), and 15 min (lanes 8 and 16). After the initiation period 38 uL aliquots were removed and added to 2 uL heparin (5 ug/mL final concentration) and the remaining nucleotide ([alpha-³²P] labeled) and allowed to elongate for 10 min. The P1 transcript was not detectable in the AGC initiation conditions. B. Transcription products were quantified as described in Methods: polymerase activity at the P2 promoter with AGC (closed circles) or GUC (closed squares) initiation conditions.

AGC

GUC

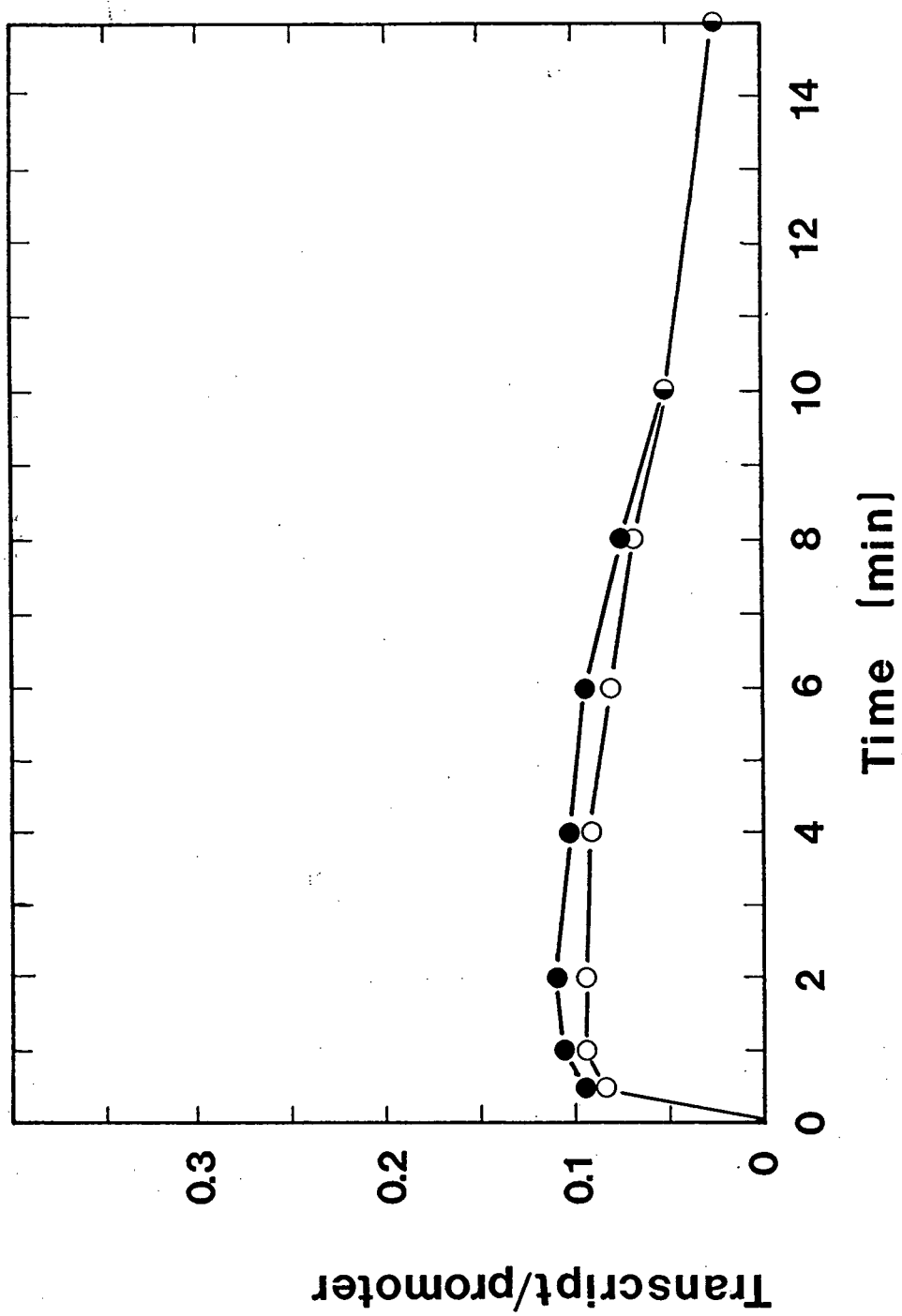




after 4 min the number of transcripts per promoter remained constant at about 0.2. When heparin resistant complexes could not be formed at P1 (AGC initiation), the formation of heparin-resistant P2 complexes was reduced by 20% at 0.5 min initiation and continued to decay throughout the duration of the experiment. This decay indicated that although heparin-resistant complexes could be formed initially at P2, the complexes did not remain resistant to heparin attack with increased initiation time. It should be noted that the decay of complexes at P2 occurred in the absence of heparin and in the presence of the nucleotides AGC.

The experiments using the tandem promoter template confirmed the initial observation made on the separated promoter templates: when the initiation mix contained the nucleotides ATP, GTP, and CTP, RNA polymerase formed heparin resistant complexes at the P2 promoter only. Initiation time course experiments using the separated P2 construct as template were performed to investigate whether the stability of the heparin-resistant complexes was similar to that observed at P2 on the tandem promoter construct. Figure 16 compares the AGC initiation data from Figure 15b to RNA polymerase activity at P2 from an initiation time course experiment where the separated P2 promoter construct was the template, the initiation mix contained AGC and the enzyme concentration was 20 nM. No significant difference in the level of heparin-resistant complex formation between the two P2 templates was seen. In addition, no significant difference in the stability of the heparin-resistant complexes was noted, i.e. decay of heparin-resistant complexes occurred on both templates.

Figure 16. The effect of P1 deletion from the rRNA promoter template on transcription initiation from P2. RNA polymerase (20 nM) was incubated with the tandem rRNA template (2.6 nM pHD1.8 DNA) (closed circles) or the separated P2 template (2.5 nM pTLXT-220 DNA) (open circles) in a solution containing 400 uM ATP, GTP and CTP for the times indicated. After the initiation period 38 uL aliquots were removed and added to 2 uL heparin (5 ug/mL, final concentration) and [alpha-³²P]-UTP (10 uM final concentration) and allowed to elongate for 10 min. Transcription products were separated by electrophoresis and quantified as described in Methods.



Unlike the activity seen at the P2 promoter, RNA polymerase activity at the P1 promoter did not change with the absence of a functional P2. Figure 17 compares the results of an initiation time course experiment in which the separated P1 construct was used as the template, the initiation mix contained GUC, and RNA polymerase was at 20 nM, with the P1 data of the tandem promoter construct in Figure 15. For both templates the level of activity rose over 4 min and remained constant, and no significant difference was noted in the level of heparin-resistant complex formation nor in the stability of the complexes. These results indicated that the formation of heparin-resistant complexes at the P1 promoter was independent of activity at the P2 promoter.

Figure 18 shows the effect of RNA polymerase concentration on the rate of heparin-resistant complex formation at P2 of the tandem promoter construct when the initiation mix contained AGC by comparing the time course of complex formation for three enzyme inputs. The decay of complexes at P2 could not be alleviated by an increase in enzyme concentration, although RNA polymerase concentration did increase the maximum level of transcription activity and the length of the incubation time at which maximum activity occurred. As the RNA polymerase concentration increased from 4 nM to 60 nM, the time to reach maximum activity increased from 1 min to 4 min. Stefano and Gralla (1979) and Dobinson and Spiegelman (1987) have reported that polymerase irreversibly denatures under initiation conditions when it cannot form stable complexes with DNA. Such denaturation probably accounts for the loss of transcription activity. The observed rate of decay from maximum to 50% maximum was about the same for the three enzyme concentrations: 6.2 min at 4 nM, 7.7

Figure 17. The effect of P2 deletion from the rRNA promoter template on transcription initiation from P1. RNA polymerase (20 nM) was incubated with the tandem rRNA template (2.6 nM pHD1.8 DNA) (closed circles) or the separated P1 template (1.5 nM pTLXT-205 DNA) (open circles) in a solution containing 400 uM GTP, UTP and CTP for the times indicated. After the initiation period 38 uL aliquots were removed and added to 2 uL heparin (5 ug/mL, final concentration) and [alpha-³²P]-ATP (10 uM final concentration) and allowed to elongate for 10 min. Transcription products were separated by electrophoresis and quantified as described in Methods.

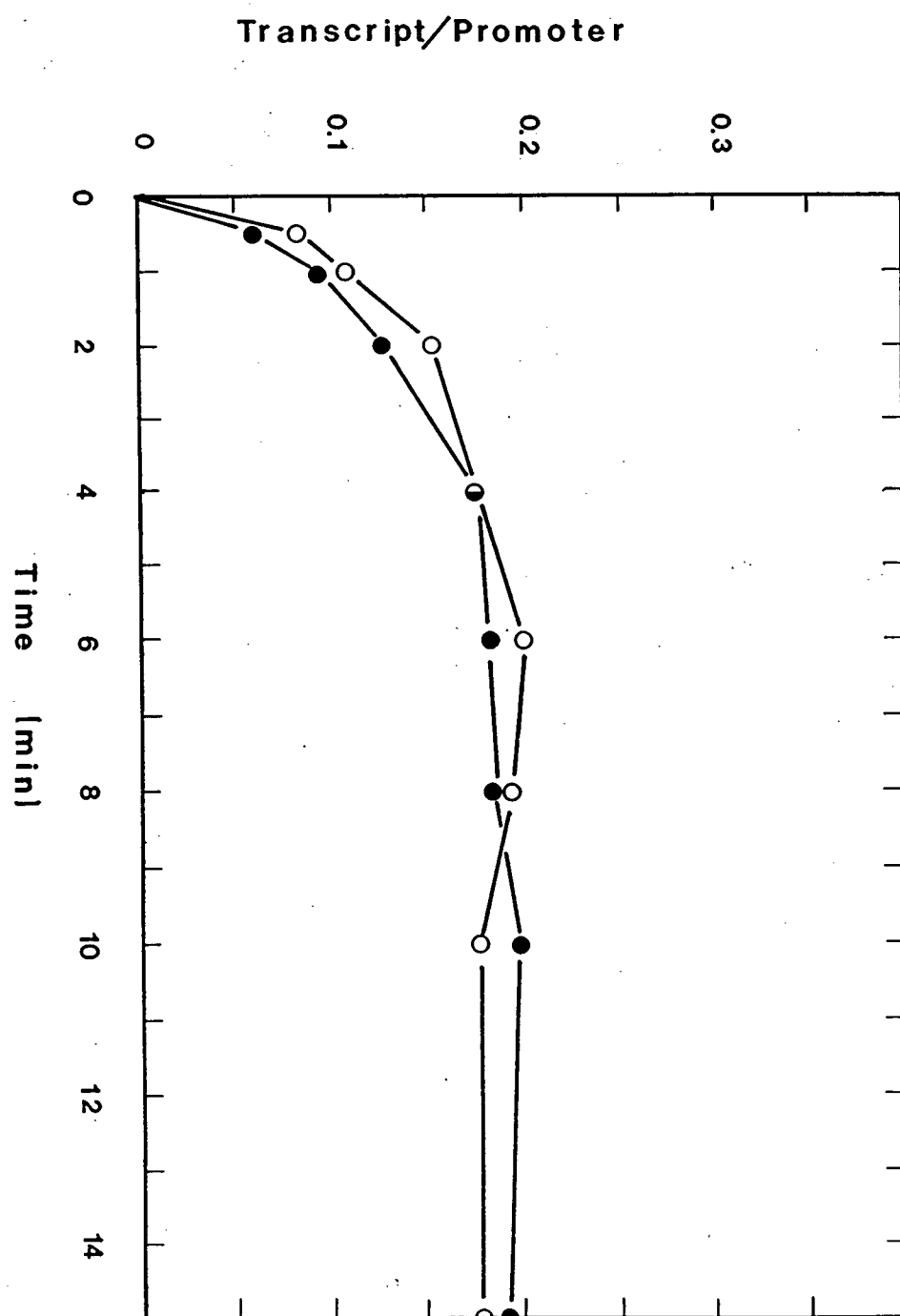
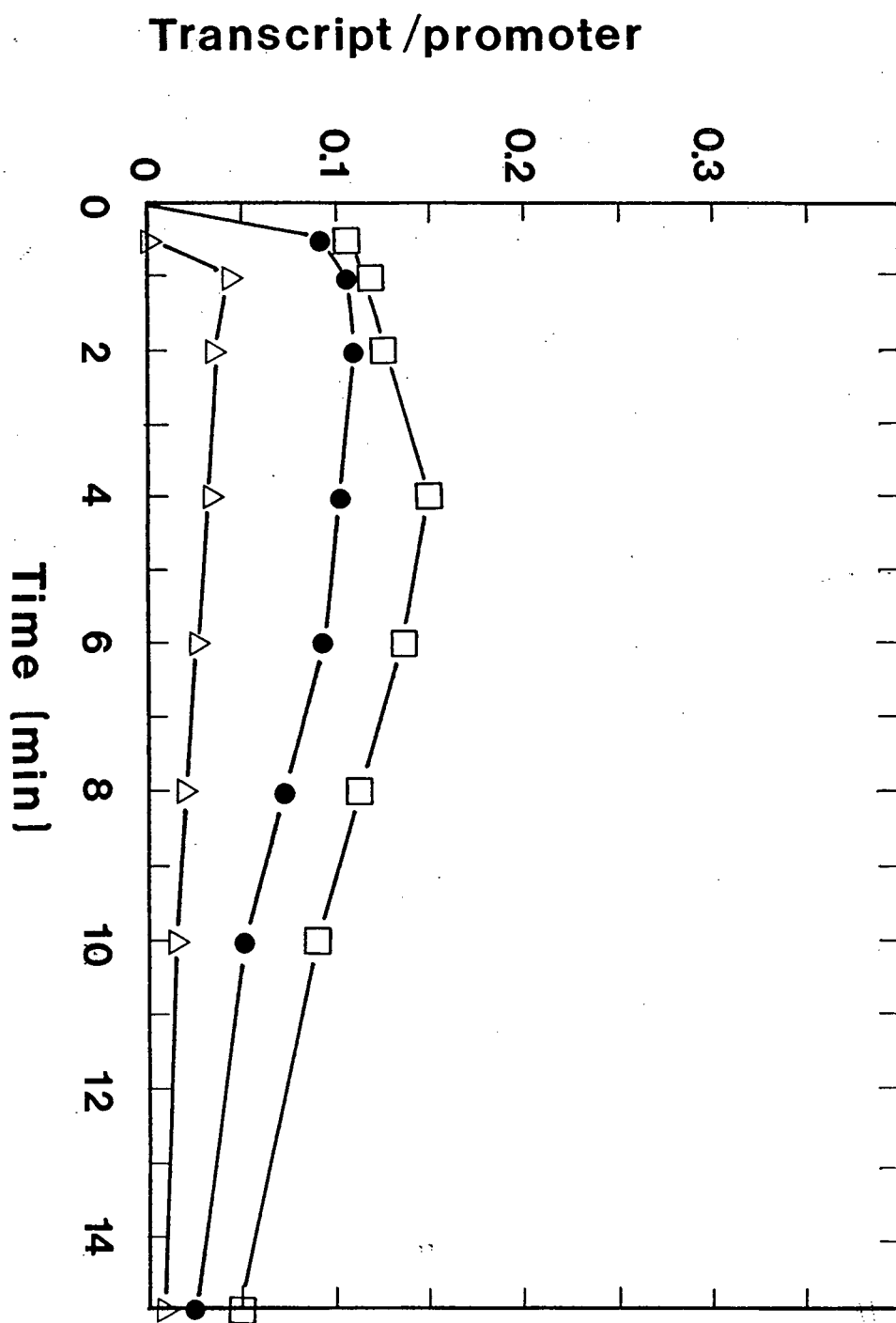


Figure 18. The effect of RNA polymerase concentration on transcription initiation at the P2 promoter of the tandem promoter rRNA template. The tandem promoter template was incubated with RNA polymerase at 4 nM (open squares), 20 nM (closed circles), and 60 nM (open triangles), in the presence of 400 uM ATP, GTP and CTP and allowed to form complexes for the times indicated. After the initiation period 38 uL were removed and added to 2 uL heparin (5 ug/mL, final concentration) and [alpha-³²P]-UTP (10 uM final concentration). Transcription products were separated by electrophoresis and quantified as described in Methods.



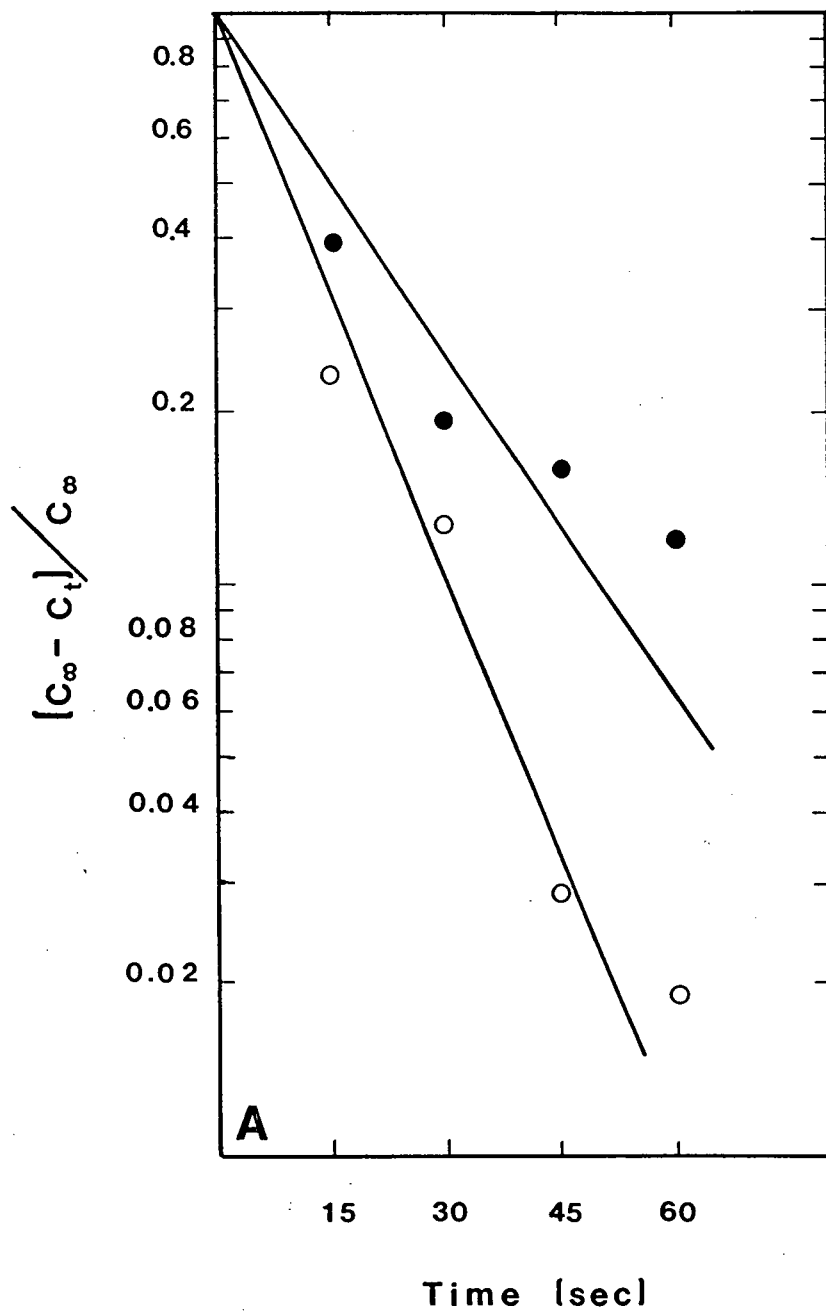
min at 20 nM, and 7.8 min at 60 nM, giving an average decay constant, $k=0.1 \text{ min}^{-1}$. The effect of enzyme concentration on the separated P2 template when the initiation mix contained AGC was the same as that shown in Figure 18 (data not shown).

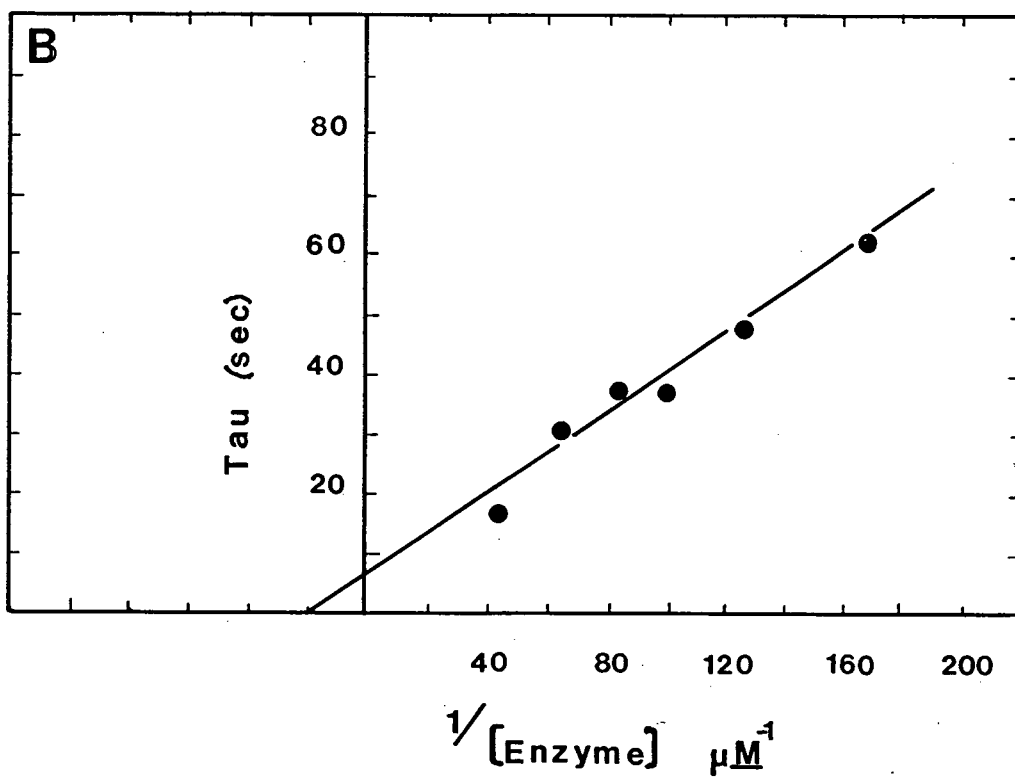
E. Kinetic analysis of tandem rRNA promoters

The initiation rate assay illustrated in Figure 13 showed that RNA polymerase at the rRNA promoters required more time to reach maximum activity than did the enzyme at the two $\phi 29$ promoters. To compare the kinetic characteristics of the polymerase at the tandem rRNA promoters to those of the enzyme at the A2 promoter, initiation rate experiments were performed over a range of enzyme concentrations for analysis using the composite rate assay (Stefano and Gralla, 1982; Dobinson and Spiegelman, 1985) a modification of the tau plot analysis (McClure, 1980). The composite rate assay makes two assumptions: first, that polymerase at a promoter proceeds through an unstable intermediate and that its conversion to a stable complex is the rate limiting step in transcription initiation; and second, under conditions of enzyme excess, the reaction will become pseudo-first order in promoter sites. The observed rate will be influenced by the stability and conversion of the unstable intermediate to the open complex. Variation of the polymerase concentration will influence the rate by changing the fractional saturation of the promoter sites with the intermediates (Stefano and Gralla, 1982).

The tau plot is formally analogous to the Lineweaver-Burk double reciprocal plot (McClure, 1980). Transformation of initiation rate data into a tau plot permits the calculation of the overall forward rate

Figure 19. Kinetic analysis of initiation complex formation at the A2 promoter. A. Semilogarithmic plot of the data from an initiation time course. The A2 promoter template (2 nM) was incubated with RNA polymerase at 6 nM (closed circles) or 16 nM (open circles) in the presence of 400 uM ATP and GTP for the times indicated. After the initiation period 38 uL were removed and added to 2 uL heparin (5 ug/mL, final concentration), CTP (400 uM, final concentration) and [α - 32 P]-UTP (10 uM final concentration). Transcription products were separated by electrophoresis and quantified as described in Methods. The ordinate represents the fraction of available promoter sites remaining after the period of initiation, calculated from the level of transcription (C_t) obtained after initiation time= t , and the maximum level of transcription (C_∞) obtained after 10 min of initiation. B. Tau plot of initiation complex formation (heparin resistant complexes) at the A2 promoter. Each point represents the tau value ($1/k_{obs}$), calculated from the initial rate of heparin resistant complex formation for the corresponding enzyme concentration. The line was calculated by linear regression. The observed rate constant for conversion of the intermediate to a heparin resistant complex (k_2) is 0.152 s^{-1} , and the apparent dissociation constant for the intermediate (K_A^*) is 50 nM.

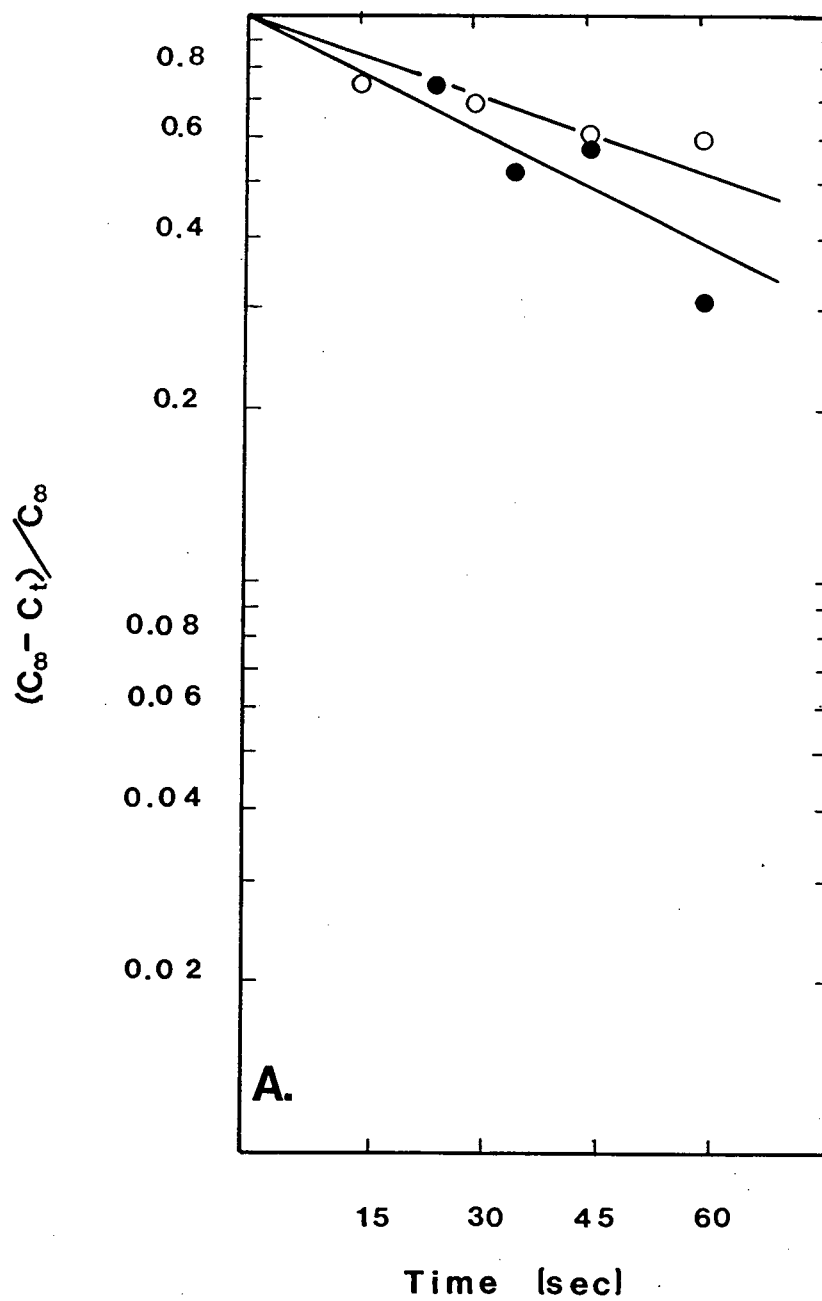




constant for the formation of initiation complexes (K_{on}), the equilibrium dissociation constant for the assumed unstable intermediate (K_A^*), and the rate constant for the conversion of the unstable intermediate to the initiated complex (k_2). Briefly, the log of the fraction of available promoter sites remaining after the initiation period was plotted against the time of the initiation period in seconds. An example of these plots is shown in Figure 19a for experiments performed at 6 nM and 16 nM RNA polymerase using the A2 promoter as template. The pseudo first order rate constant (K_{obs}) for each enzyme concentration was calculated from the slope of the resulting line. Tau values ($1/K_{obs}$) were plotted against the reciprocal of the enzyme concentration to produce the tau plot (Figure 19b). The overall forward rate constant (K_{on}), $3.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, was derived from the slope of the line; the equilibrium dissociation constant for the assumed unstable intermediate (K_A^*) was 50 nM, and calculated from the x-intercept; and the rate constant for the conversion of the unstable intermediate to the initiated complex (k_2) was 0.152 s^{-1} , and calculated from the y-intercept. Dobinson reported similar values at the A2 promoter for RNA polymerase preparations which had low delta content (Ph. D. Thesis, University of British Columbia, 1986).

Initiation rate experiments were performed using the tandem promoter construct as template and an initiation mix containing GUC. Figure 20 shows examples of the semilogarithmic plot of the fraction of remaining promoter sites available after the initiation period at 16 nM and 24 nM RNA polymerase. Unlike the A2 results the slope of the line, the pseudo first order rate constant (K_{obs}), decreased with increased enzyme concentration for both P1 (Figure 20a) and P2 (Figure 20b). When the tau values ($1/K_{obs}$) were plotted against the reciprocal of the enzyme

Figure 20. Semilogarithmic plot of the data from an initiation time course with the tandem rRNA promoter template. The tandem promoter template (2.6 nM pHD1.8 DNA) was incubated with RNA polymerase at 16 nM (closed circles) or 24 nM (open circles) in the presence of 400 uM GTP, UTP, and CTP for the times indicated. After the initiation period 38 uL were removed and added to 2 uL heparin (5 ug/mL, final concentration) and [alpha-³²P]-ATP (final concentration 10 uM). Transcription products were separated by electrophoresis and quantified as described in Methods. The ordinate is the same as in Figure 19a. Data from the P1 promoter are shown in panel A and data from the P2 promoter are shown in panel B.



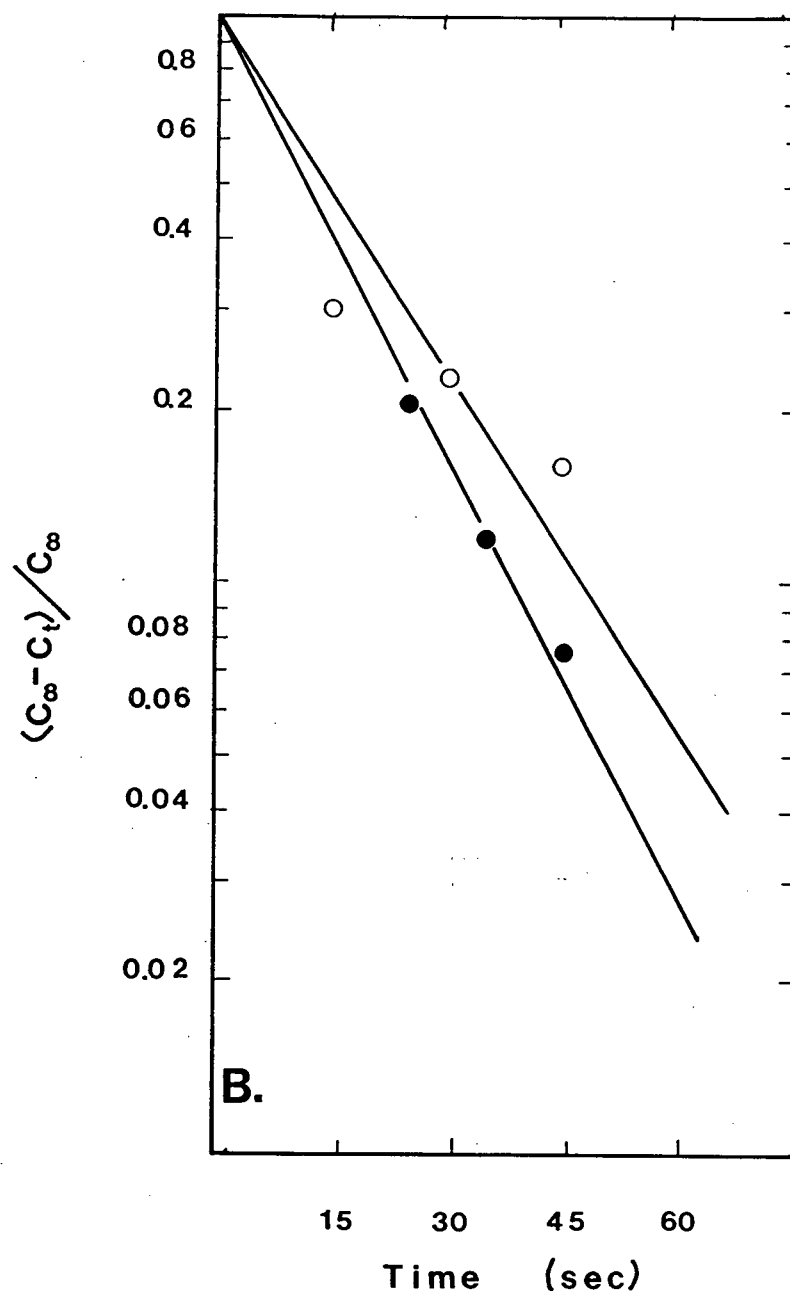
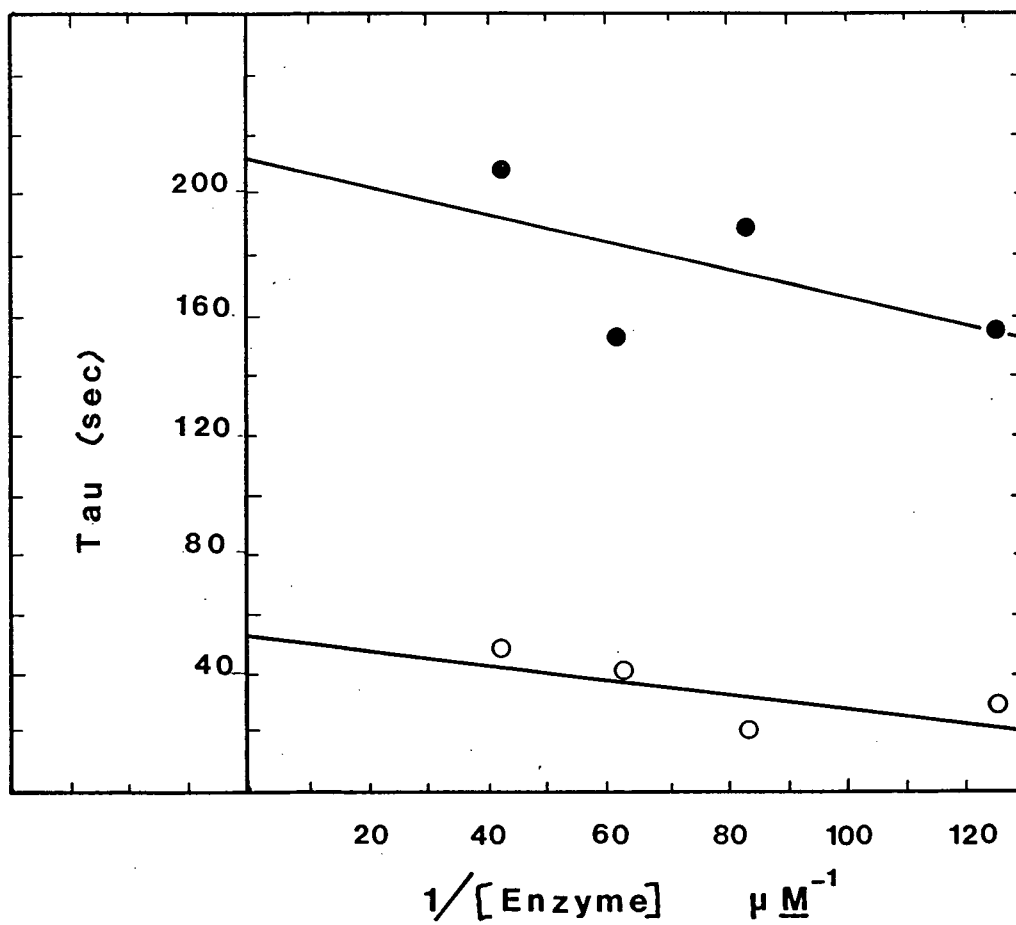


Figure 21. Tau plot of initiation complex formation (heparin resistant complexes) at the P1 (closed circles) and P2 (open circles) promoters. Each point represents the tau value ($1/k_{obs}$), calculated from the initial rate of heparin resistant complex formation for the corresponding enzyme concentration. The lines were calculated by linear regression.



concentration, flat or slightly negative slopes were obtained from the data from both rRNA promoters (Figure 21), indicating that the K_{on} and K_A^* for the rRNA promoters could not be calculated by this method. However, the rate constant for the conversion of the unstable intermediate to the initiated complex, k_2 , could be calculated from the y-intercept: for P1 $k_2=0.005\text{ s}^{-1}$, while for P2 $k_2=0.019\text{ s}^{-1}$. The k_2 values for the rRNA promoters are 30 and 7.5 times slower, respectively, than those obtained for the A2 promoter and might account for the slow initiation rates observed in Figure 13.

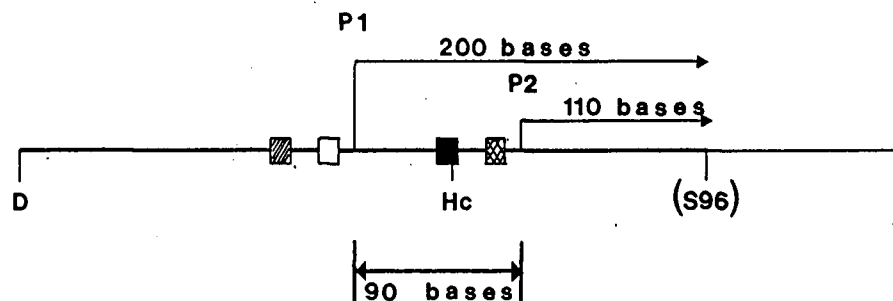
F. Formation of heparin resistant complexes on mutant constructs

The data presented in Figures 14, 15, and 16 suggested that in order for RNA polymerase to form stable, heparin resistant complexes at P2, heparin resistant complexes must also be formed at P1. To further investigate the relationship between the complexes formed at P1 and P2 in the native configuration, initiation rate experiments were performed using two mutant tandem promoter constructs.

The plasmid pPldP2 was used to investigate the effect of increasing the distance between P1 and P2 on the formation of heparin resistant complexes at both promoters. Figure 22 describes the procedure used in the construction of pPldP2. Briefly, a DNA fragment containing the isolated P2 promoter was cloned into the BamHI site of a plasmid containing the isolated P1 promoter, maintaining the orientation but increasing the distance between P1 and P2 from 90 bases to 185 bases (C. Brion, personal communication).

Figure 22. Structure of the wild type promoter region and an insertion mutant of the rrnB operon. A. The promoter region of plasmid pKK427B which contains the native promoter region of the rrnB operon and is redrawn from Figure 8. B. The promoter region of plasmid pPldP2 which contains a 95 base insert in the promoter region of the rrnB operon. Plasmid pPldP2 was constructed by inserting the 220 base pair fragment which contains only the P2 promoter from pKK220 (similar to pTLXT220 shown in Figure 8) into the BamHI site of pKK282B, which contains only the P1 promoter (personal communication C. Brion). The single hatched box represents the -35 region of the P1 promoter; the open box represents the -10 region of the P1 promoter; the filled box represents the -35 region of the P2 promoter; and the double hatched box represents the -10 region of the P2 promoter. Restriction sites are designated as follows: BamHI (B), Sau96I (S96), DdeI (D), and HincII (Hc). Restriction sites in parenthesis have been destroyed by cloning but are shown for reference. The lengths of the runoff transcripts produced in in vitro transcription reactions are indicated.

A. pKK427B- native construct



B. pP1dP2- increased distance construct

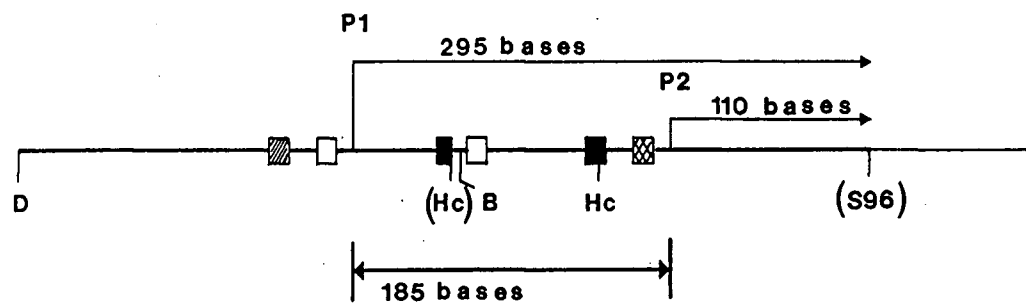
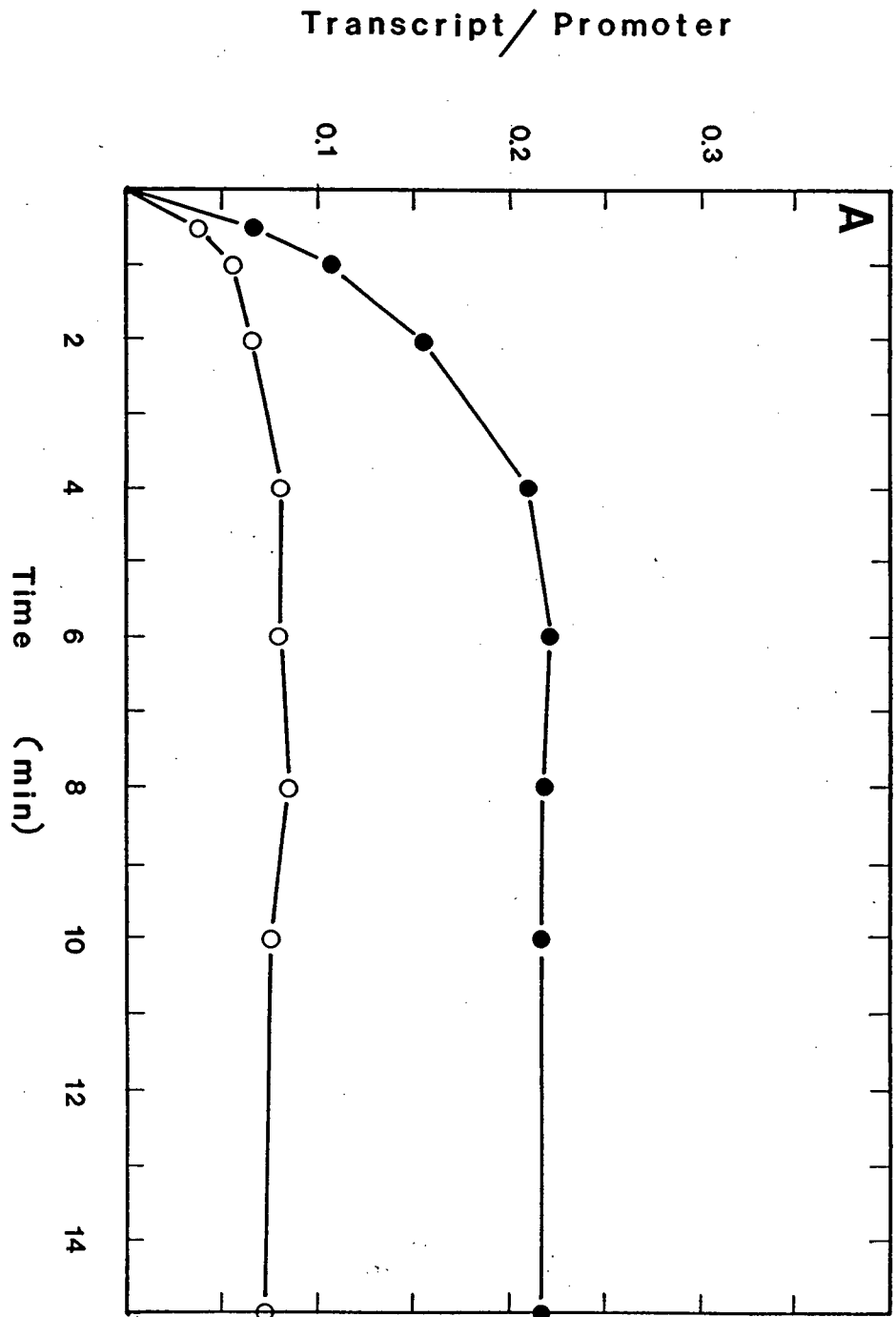


Figure 23 shows the averaged results of a number of initiation time course experiments in which RNA polymerase at 20 nM was incubated with either the native tandem promoter template or the increased distance tandem promoter template in an initiation mix containing GUC. Since the rate of transcription initiation at the P1 promoter was found to be the same on the separated and tandem promoter templates (Figure 17), the initial prediction was that increasing the distance between rRNA promoters would affect polymerase activity at P2, but not at P1. However, the number of heparin resistant complexes formed at both promoters declined when the distance between them was increased. At P1 of the mutant construct the plateau level of polymerase activity was reduced by almost 3 times when compared to the activity at P1 of the native construct (Figure 23a), while the plateau level of activity at P2 was about 1.6 times lower on the mutant template than on the native template (Figure 23b).

Additional initiation rate experiments performed at 4 nM and 60 nM RNA polymerase with pPldP2 or pHD1.8 as templates indicated that the relative levels of polymerase activity at the promoters in the mutant construct were not constant when compared to activity at the promoters on the native construct. In Table IV the relative activity of the polymerase at P1 and P2 of the mutant construct is expressed as a percent of the plateau level of activity at the respective promoters in the native construct. As the enzyme concentration increased the polymerase activity at the promoters on the mutant construct approached the level observed at the promoters on the native construct.

Figure 23. The effect of increasing the distance between the P1 and P2 promoters on transcription initiation. A. The effect of initiation time on transcription initiation from the P1 promoter of pHD1.8 (closed circles) and the P1 promoter of pPldP2 (open circles). B. The effect of initiation time on transcription initiation from the P2 promoter of pHD1.8 (closed circles) and the P2 promoter of pPldP2 (open circles). The data from the pHD1.8 promoters (closed circles) are the average of three initiation time course experiments in which the tandem promoter template (2.6 nM) was incubated with RNA polymerase (20 nM) in the presence of 400 uM GTP, UTP, and CTP for the times indicated. After the initiation period 38 uL were removed and added to 2 uL heparin (5 ug/mL, final concentration) and [α -³²P]-ATP (final concentration 10 uM). Transcription products were separated by electrophoresis and quantified as described in Methods. The data from pPldP2 (open circles) are from the average of two initiation time course experiments in which the increased distance template (1.5 nM) was incubated with RNA polymerase (20 nM) under the same conditions as described for pHD1.8.



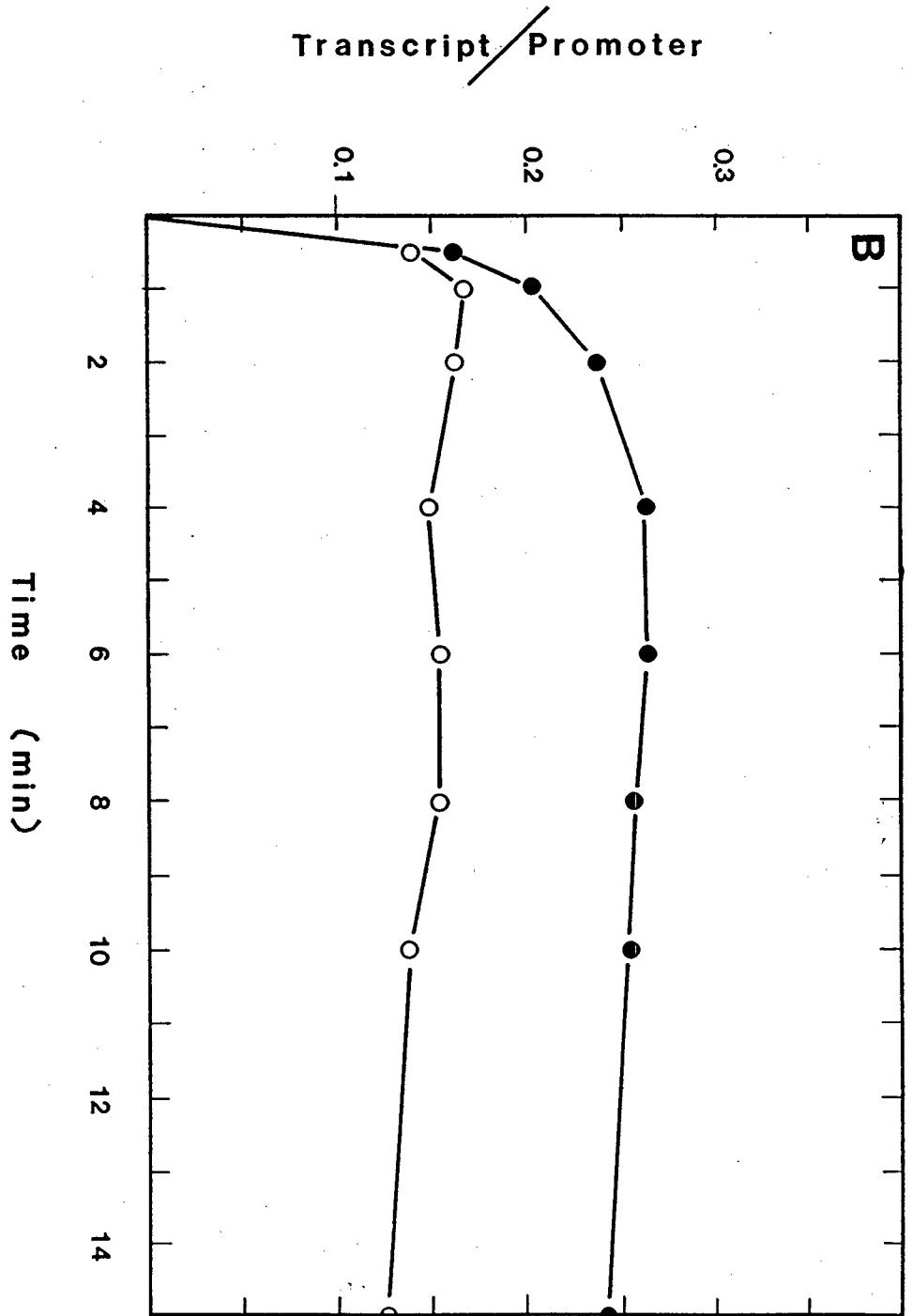


TABLE IV

Relative RNA polymerase activity at
P1 and P2 promoters of pPldP2

| RNA polymerase nM | % Activity on Native Template ^a | |
|----------------------|--|----|
| | P1 | P2 |
| 4 | 10 | 21 |
| 20 | 35 | 60 |
| 60 | 80 | 92 |

^a For experiments performed at each RNA polymerase concentration the plateau level of polymerase activity at P1 and P2 of the mutant construct was divided by the plateau level of activity at P1 and P2 of the native construct and multiplied by 100.

The results presented in Figure 23 and Table IV suggested that the interaction between the rRNA promoters was more complex than initially assumed. The cloning scheme used to make pPldP2 involved the ligation of two DNA fragments which contained the sequences for the -35 region of P2 and the -10 region of P1 such that a promoter-like region between P1 and P2 was created (see Figure 22). The results from these initial rate experiments could not determine whether the reduced polymerase activity observed at the promoters on pPldP2 was due solely to the increased distance between the tandem promoters or whether the increase in rRNA promoter region sequences also affected the results.

The plasmid pPlA2 (Figure 24) was used as a template to investigate whether the presence of the rRNA P1 promoter upstream of the ϕ 29 A2 promoter would affect polymerase activity at the downstream promoter. Plasmid pPlA2 was constructed by cloning a 165 base pair fragment containing the A2 promoter into the BamHI site of a plasmid which contained the separated P1 promoter (C. Brion, personal communication). The distance between the artificial tandem promoters is 126 bases (C. Brion, personal communication), rather than the 90 bases seen in the native rRNA construct.

Figure 25a is an autoradiogram of a initiation rate experiment in which RNA polymerase at 20 nM was incubated with pPlA2, linearized downstream of the A2 promoter, in a mix containing either GUC (lanes 1-8) or AG (lanes 9-16). The results showed that the initiation conditions required for the formation of heparin resistant complexes at the promoters on the artificial tandem promoter template, were the same as those that produced heparin resistant complexes on the respective native

Figure 24. Structure of the tandem promoter region of pPlA2. The tandem promoter region of the plasmid pPlA2 contains the P1 promoter of the rrnB operon and the A2 promoter of ϕ 29. Plasmid pPlA2 was constructed by inserting the 165 base pair fragment containing the A2 promoter into the SalI site of pKK282B, which contains only the P1 rRNA promoter (personal communication, C. Brion). Restriction sites are designated as follows: BamHI (B), DdeI (D), and HindIII (Hd). The lengths of the runoff transcripts produced in in vitro transcription reactions are indicated. The distance between the two promoters is 126 base pairs.

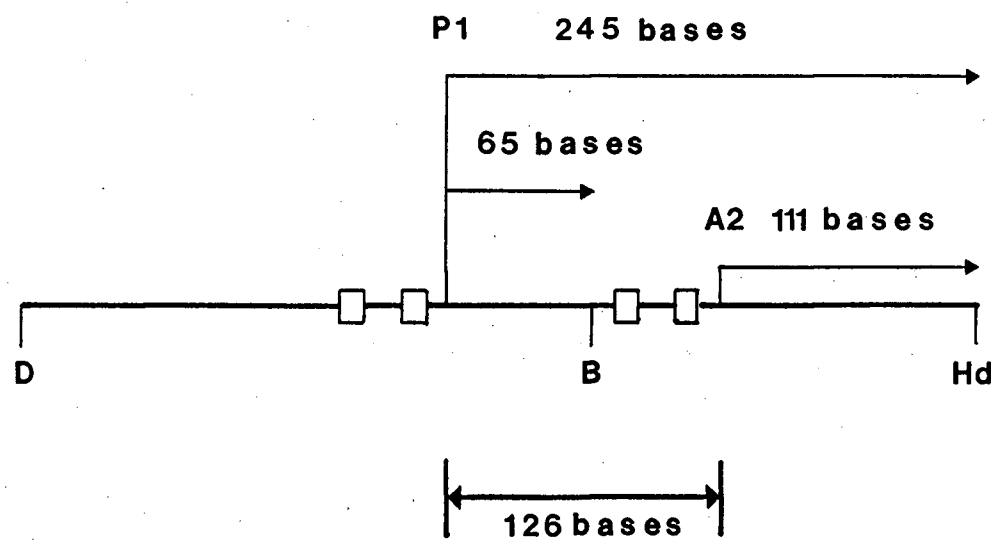
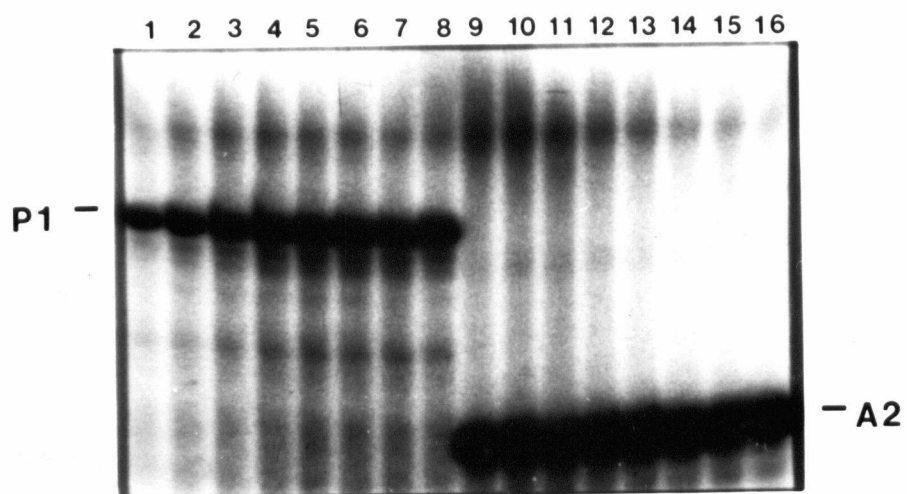
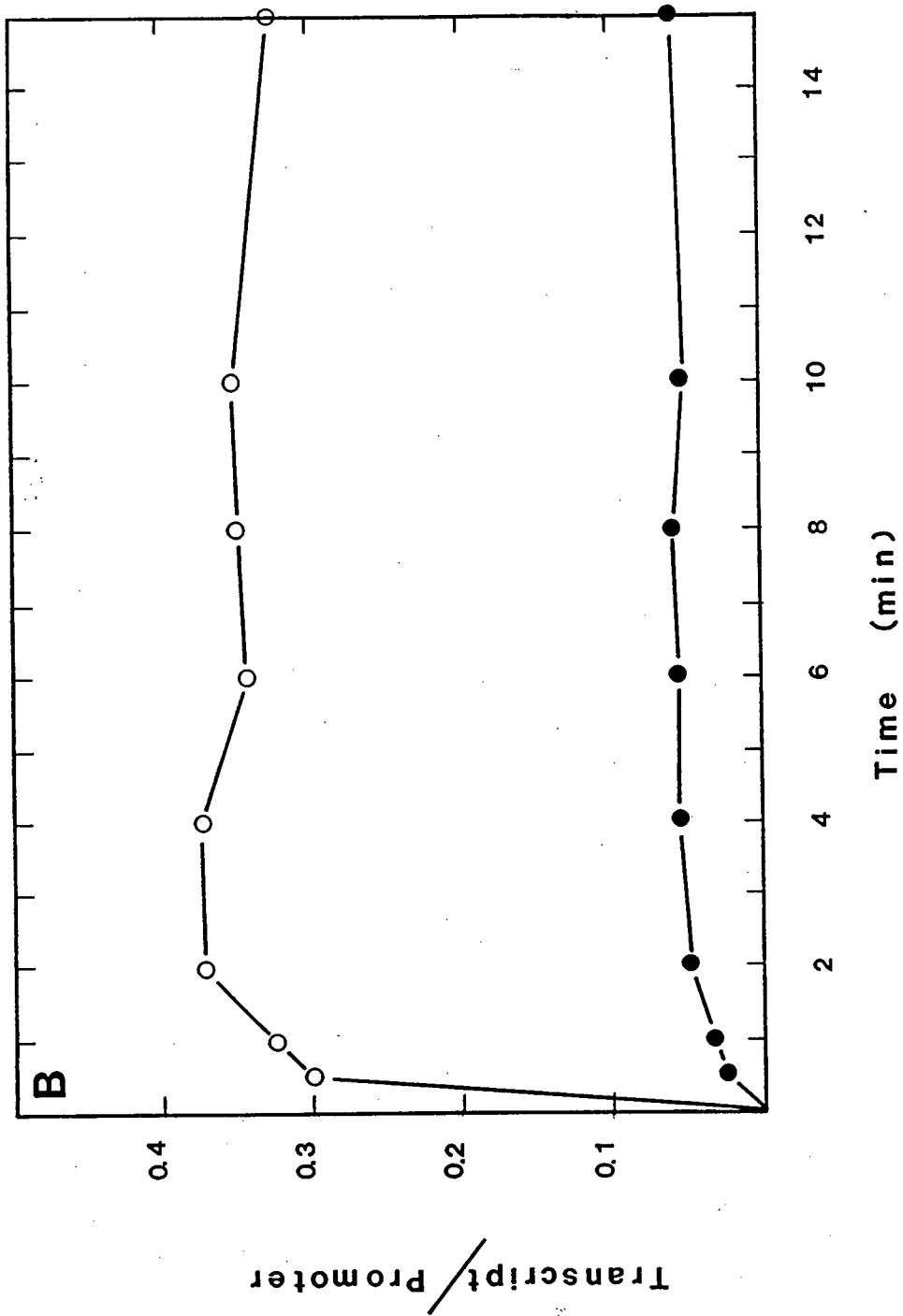
_pP1A2

Figure 25. The effect of initiation nucleotides on transcription initiation from the tandem promoters of pPlA2. A. Autoradiogram of polyacrylamide gel of transcription products. RNA polymerase (20 nM) was incubated with the pPlA2 tandem promoter template (2.1 nM) in the presence of 400 uM GTP, UTP and CTP (lanes 1-8) or 400 uM ATP and GTP (lanes 9-16). Initiation periods were for 0.5 min (lanes 1 and 9), 1 min (lanes 2 and 10), 2 min (lanes 3 and 11), 4 min (lanes 4 and 12), 6 min (lanes 5 and 13), 8 min (lanes 6 and 14), 10 min (lanes 7 and 15), and 15 min (lanes 8 and 16). After the initiation period 38 uL aliquots were removed and added to 2 uL heparin (5 ug/mL final concentration) and the remaining nucleotide(s): [alpha-³²P]-ATP (10 uM final concentration) lanes 1-8; or CTP and [alpha-³²P]-UTP (400 uM and 10 uM final concentration, respectively) lanes 9-16; and allowed to elongate for 10 min. B. Transcription products were quantified as described in Methods: RNA polymerase activity at the P1 promoter with GUC initiation conditions (closed circles) and the A2 promoter with AG initiation conditions (open circles).

GUC

AG



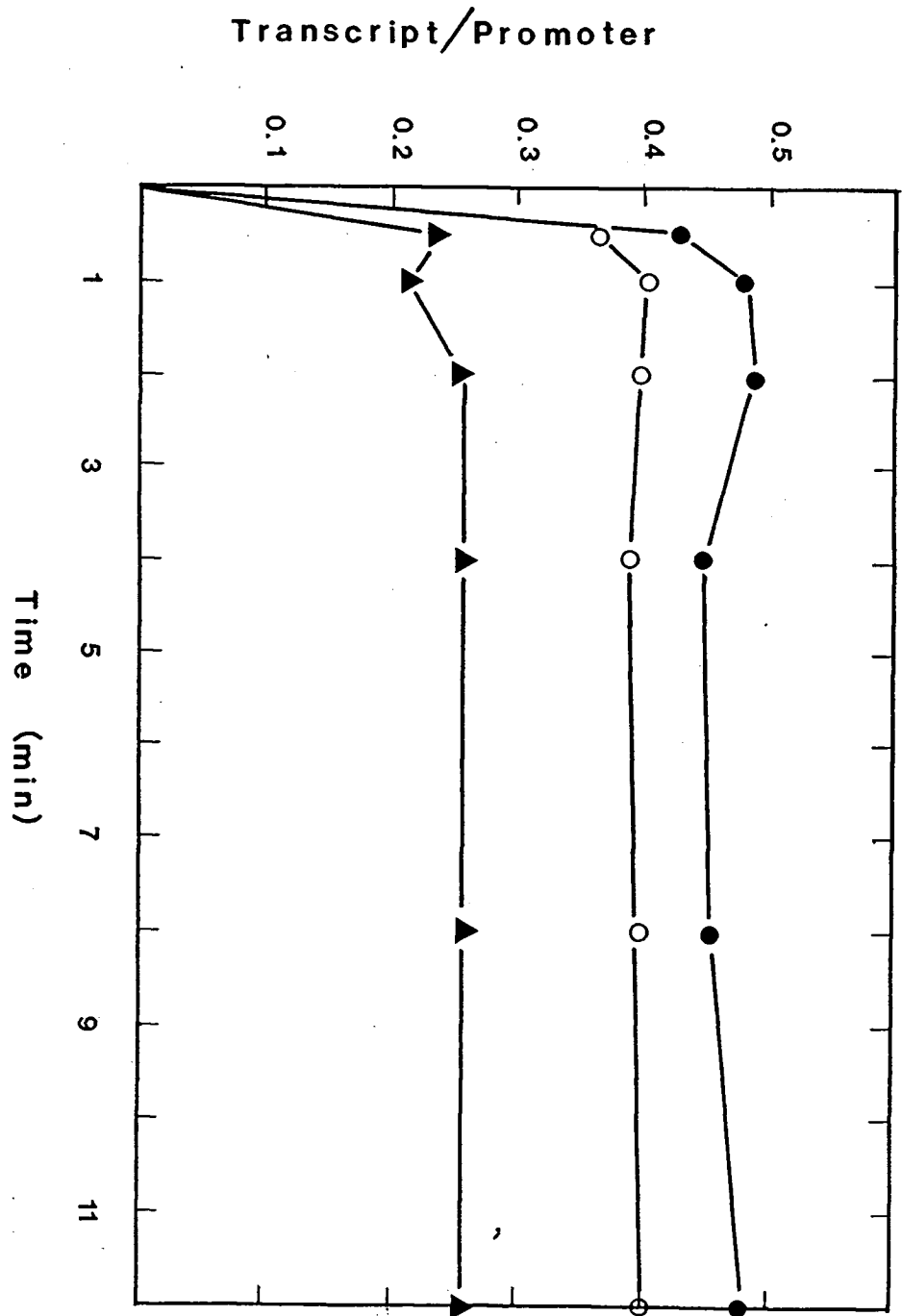


templates (Dobinson and Spiegelman, 1987; Figures 13, 14, and 15 above). When the number of transcripts/promoter was calculated (Figure 25b), the level of polymerase activity at the P1 promoter was about 2.5 times lower than that observed at P1 on the native template (see Figure 13), while the level of activity at the A2 template was over 2 times higher than the activity at A2 on the native template (see Figure 13). Although the presence of P1 upstream of A2 did not alter the requirements for RNA polymerase to form heparin resistant complexes at A2, the presence of P1 upstream of A2 did appear to increase polymerase activity at A2.

To further investigate the effect of the presence of the P1 promoter upstream of the A2 promoter on polymerase activity at A2, a series of initiation rate experiments were performed using as templates various restriction endonuclease digests of pPlA2 which differentially isolated the promoters. Figure 26 displays the results of one such experiment, where polymerase was incubated with pPlA2 digested with either HindIII, which cleaves the plasmid downstream of the A2 promoter leaving P1 and A2 attached, or both HindIII and BamHI, which removes the P1 promoter from the region upstream of the A2 promoter (each at 2.1 nM), as template. In control reactions polymerase was incubated with EcoRI digested p328-5 (at 2.0 nM), which contains the native A2 promoter. All reactions included AG in the initiation mix.

Figure 26 shows that RNA polymerase activity was lowest at the A2 promoter on the control template, a plateau level of about 0.26 transcripts per promoter being attained. Polymerase activity at the A2 promoter when the P1 promoter was physically linked (HindIII digested template), was 1.8 times greater than the control. However, activity at

Figure 26. Time course of transcription initiation at the A2 promoter when the P1 rRNA promoter is upstream. RNA polymerase (20 nM) was incubated with the pPlA2 template digested HindIII (P1 upstream of A2, see Figure 24; closed circles), the pPlA2 template digested with BamHI and HindIII (P1 not upstream of A2, see Figure 24; open circles) or the p328-5 template digested with EcoRI (A2 promoter in native environment, see Figure 9, closed triangles), in the presence of 400 uM ATP and GTP for the time indicated. After the initiation period 38 uL aliquots were removed and added to 2 uL heparin (5 ug/mL, final concentration) and the remaining nucleotides, CTP and [α -³²P]-UTP (400 uM and 10 uM final concentration, respectively). Transcription products were separated by electrophoresis and quantified as described in Methods. The concentration of the templates was 2.1 nM for pPlA2 and 2 nM for p328-5.

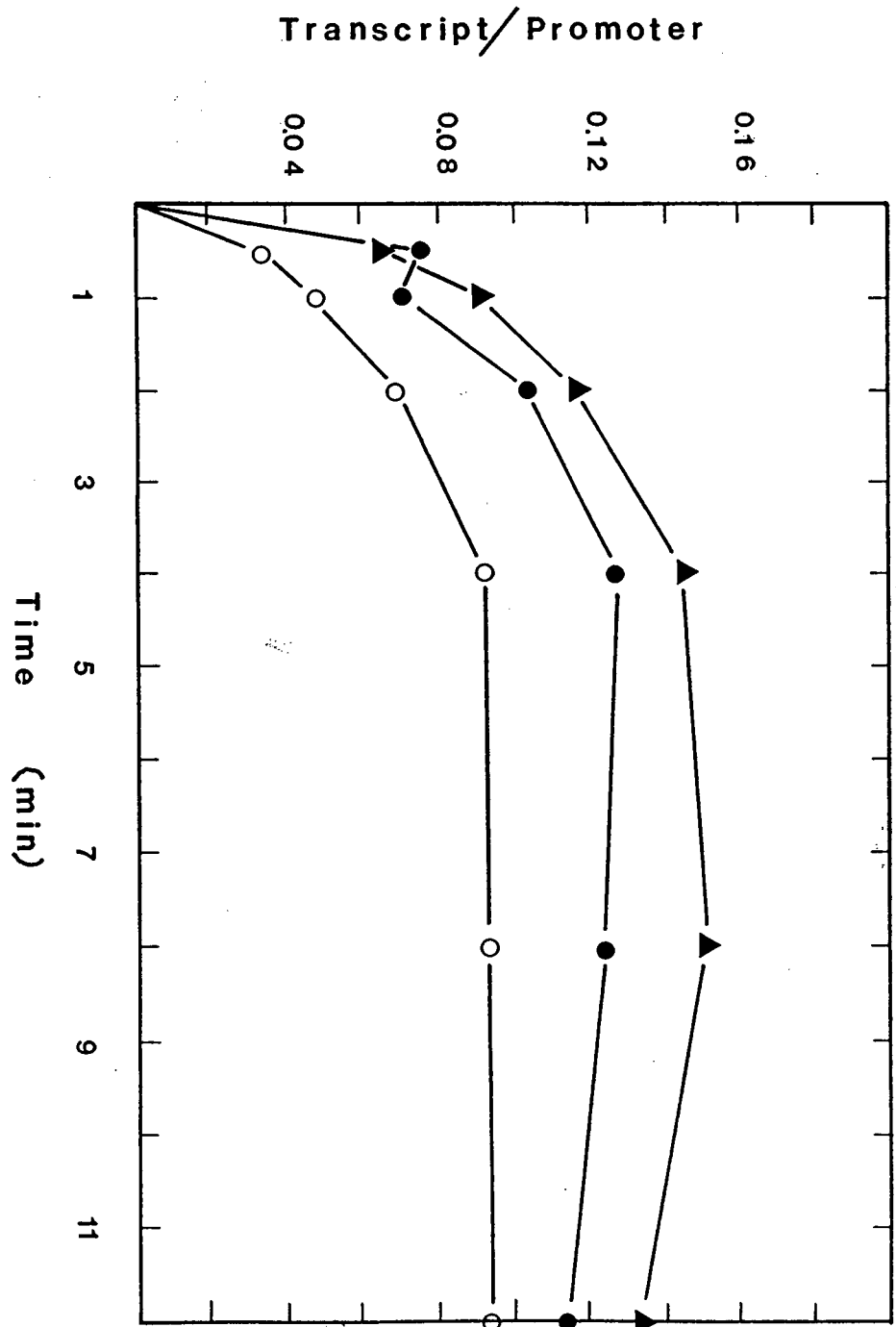


the A2 promoter when the P1 promoter was no longer upstream (the double digested template) also increased by 1.5 times. The removal of some inhibitory sequences when the DNA fragment containing the A2 promoter was subcloned to produce pPlA2 might account for the increased polymerase activity at A2 on the double digested template. Nevertheless, when the P1 promoter was physically linked to the A2 promoter (HindIII digested template) polymerase activity at the A2 promoter increased 30%, and unlike the situation at the P2 promoter, a heparin resistant complex at the P1 promoter was not required for an effect at the A2 promoter.

Figure 27 presents the results of an experiment using pPlA2 designed to investigate the effect of the presence of the A2 promoter downstream on polymerase activity at the P1 promoter. The figure shows the results of an initiation rate experiment in which RNA polymerase (20 nM) was incubated with pPlA2 digested with either BamHI, which cleaved the plasmid DNA between the P1 promoter and the A2 promoter, or HindIII, which cleaved the plasmid downstream of the A2 promoter (each at 2.1 nM), as template. The separated P1 promoter construct, pTLXT-205 (at 1.5 nM), digested with BamHI was the control template and all reactions contained GUC in the initiation mix.

The results from Figure 27 indicated an apparent decrease (to 85% of the pTLXT-205 control) in RNA polymerase activity at the P1 promoter when the A2 promoter was not physically downstream (BamHI digested pPlA2 template). This apparent decrease in activity could be due differences in enzyme to promoter ratios. Theoretically there were 13 polymerase molecules for every P1 promoter when the separated promoter construct served as template and 5 polymerase molecules for every P1 promoter when

Figure 27. Effect on transcription initiation at the P1 promoter when the Φ 29 A2 promoter is downstream. RNA polymerase (20 nM) was incubated with the pPlA2 template digested with HindIII (A2 downstream of P1, see Figure 24; open circles), the pPlA2 template digested with BamHI (A2 not downstream of P1, see Figure 24; closed circles), or pTLXT205 template digested with BamHI (P1 promoter in native environment, see Figure 8; closed triangles), in the presence of 400 μ M GTP, UTP, and CTP for the times indicated. After the incubation period 38 μ L aliquots were removed and added to 2 μ L heparin (5 μ g/mL, concentration) and [α - 32 P]-ATP (10 μ M, final concentration). Transcription products were separated by electrophoresis and quantified as described in Methods. The concentration of the templates was 2.1 nM for pPlA2 and 1.5 nM for pTLXT205.



BamHI digested pPlA2 was the template, assuming that RNA polymerase interacted with the A2 promoter. The assumption that polymerase interaction was with the A2 promoter rather than non-specific DNA is supported by the fact that the actual DNA concentrations were about the same for all reactions (0.28 - 0.3 ug/mL). Further, when the A2 promoter was physically linked to the P1 promoter (the HindIII digested pPlA2 template), RNA polymerase activity at the P1 promoter was reduced by 30% when compared to activity on the unlinked pPlA2 template (BamHI digested). Taken together the data from Figures 25, 26 and 27 suggested that when the P1 and A2 promoters were not physically linked RNA polymerase activity at one promoter was independent of activity at the other promoter; however, when the promoters were linked, P1 appeared to stimulate polymerase activity at A2.

G. Summary of in vitro results

The results presented in this section showed the following:

1. Two approaches were taken to isolate an rRNA specific RNA polymerase: affinity chromatography and zonal centrifugation. In both cases when purified enzyme fractions were tested on rRNA and non-rRNA promoters, no difference in specificity was observed.
2. In single round transcription assays where the effect of polymerase concentration was investigated, a greater enzyme concentration was required for maximum activity at the rRNA promoters than at the ϕ 29 A2 promoter. In initiation rate experiments where the effect of the initiation period was studied, RNA polymerase required a longer period to reach

maximum activity at the rRNA promoters than at the $\phi 29$ A2 and G2 promoters.

3. When RNA polymerase could form heparin resistant complexes at the P1 promoter, the formation of heparin resistant complexes at the P2 promoter was stable. However, when RNA polymerase could not form heparin resistant complexes at the P1 promoter, either because of unfavorable initiation conditions or due to deletion, the heparin resistant complexes formed at the P2 promoter were not stable.

4. Initiation rate experiments were performed using the tandem rRNA as template for kinetic analysis in a modified tau plot. The initiation rate at both rRNA promoters did not increase with increased enzyme concentration as it did for the $\phi 29$ A2 promoter, thus the overall forward rate constant and the equilibrium dissociation constant could not be determined using the modified tau plot analysis. However, the rate constant for the conversion of the unstable intermediate to the initiated complex could be calculated, and was found to be relatively slow.

5. Two artificial tandem promoter constructs were used to investigate parameters important for interactions between the tandem promoters on the native construct. When the A2 promoter was not linked to P1 on pPlA2 template, polymerase activity at the P1 promoter was about the same as activity at the P1 promoter on the separated construct control template. Further, when the P1 promoter was upstream of the A2 promoter, polymerase activity was stimulated at the A2 promoter. However, activity at both the P1 and P2 promoters on pPldP2 decreased. These results

suggested that the interactions between tandem promoters were complex and that more suitable constructs should be used in further investigations.

V. DISCUSSION

A. Ribosomal RNA synthesis during steady state growth and nutritional shift-up in *B. subtilis*

The percent rRNA synthesis under various culture conditions was measured by hybridization of pulse labeled RNA to plasmid DNA containing the 3' end of the 23S RNA gene and the 5S RNA gene of *B. subtilis*. The hybridization efficiencies were uniformly high and the reproducibility of the reaction was such that changes in the percent rRNA synthesis as low as 5% could be identified. DNA saturation curves reached plateau levels with less than 16 ug DNA per reaction, that value represents a 7-fold excess over the RNA added to the hybridization reaction. Over the growth range $\mu=0.68$ to $\mu=1.98$ rRNA synthesis showed an increase of 43% to 63% of the total RNA synthesized with an increase of 12%/u (Figure 5). These values agree closely with those reported by Gausing (1977, 1980) for *E. coli* within the same growth rate range. Data reported by Shepherd et al. (1980), also for *E. coli*, collected by hybridization of pulse labeled RNA to specific probes, exhibit a similar trend but are somewhat higher; it is not clear why the results are different.

When a culture of *B. subtilis* growing on an acetate based medium was subjected to a shift-up in growth conditions by the addition of glucose, casamino acids, and adenosine, the rate of rRNA synthesis increased rapidly and within 10 min was 10% of the final adjusted rate (Figure 6). The final level of increase in percent rRNA transcription is similar to that measured in *E. coli* by Gausing (1977) and Zengel and

Lindahl (1986), however, both E. coli studies described different kinetics for the rate of increase in rRNA synthesis. For example, Zengel and Lindahl (1986) reported that the relative rate of rRNA synthesis increased to about 1.5 times the initial rate within 30-60 sec after the shift-up, then dropped to about 1.2 times between 2-4 min after the shift, and reached a final level of about 1.35 times at 10 min after the shift.

There are two technical factors why such fluctuations and early plateau level might not have been observed in the data presented in Figure 6. First, the time between points and the duration of the pulse label in the experiment illustrated in Figure 6 were relatively long in comparison to the 24 sec pulse with samples taken every 20-30 sec in the E. coli studies. Both of these factors would reduce the sensitivity of my assay. An estimate of 15 min as the time at which the final rate of synthesis was reached was made by determining the intersection of the initial rate line and the plateau line. Second, the DNA probe used in this study was missing about 2 kb from the 5' end of the ribosomal operon (Figure 1) while the probes used in the E. coli studies included the 5' end of the operon. Two kilobases of rRNA can be synthesized in less than 30 sec in E. coli at 37° C (Bremer and Dennis, 1987), thus the estimated time at which the final rate of synthesis was reached in the experiment shown in Figure 6 can be calculated as about 14.5 min after the shift-up.

Aside from the above technical reasons, it is possible that the slower response time in the B. subtilis system is due to intrinsic differences between the Gram negative, enteric, facultative anaerobe E. coli, and the Gram positive, spore forming, aerobe B. subtilis. From

a teleological point of view, E. coli must respond quickly to changes in the environment, whereas, B. subtilis always has the option of spore formation.

B. Using SP01 to probe RNA polymerase partitioning

The availability of RNA polymerase for distribution between rRNA and non-rRNA promoters was the primary focus of the in vivo study of rRNA synthesis in B. subtilis. The percent of active RNA polymerase has been shown to increase from about 10% in slow growing cells to about 40% in fast growing cells for both B. subtilis (Leduc, et al., 1982) and E. coli (Shepherd, et al., 1980). When a culture undergoes a nutritional shift-up an immediate increase in the percent rRNA synthesis is observed (Figure 6; Gausing, 1980; Zengel and Lindahl, 1986), suggesting that redistribution of existing polymerase, rather than de novo synthesis, is the primary mechanism for the increase (Maaloe and Kjeldgaard, 1966; Dennis and Bremer, 1974; Nierlich, 1978; Gausing, 1980). Infection with the large HMU containing DNA phages of Bacillus leads to specific and temporal modifications of the cellular RNA polymerase (reviewed in Doi and Wang, 1986). The bacteriophage SP01 was used to probe RNA polymerase availability for transcription of rRNA operons during a nutritional shift-up.

In preliminary experiments it was shown that phage infection had a varied effect on the rate of host nutrient transport (Figure 2). The rate of methionine transport was unaffected by phage infection and the rates of glucose and leucine transport were only mildly affected, 90% and 75% of uninfected rates respectively. Since carbon and nitrogen sources

are the primary effectors in a nutritional shift-up, it was concluded that phage infection would not preclude the shift-up response at the level of transport of these nutrients.

The results of the transport assay, however, indicated that the rates of nucleoside and base transport were affected by SP01 infection. The rates of cytidine, adenosine, and adenine transport were reduced to 40% of the uninfected rates and the rate of uridine transport was reduced to 60%. Beaman, et al. (1983) reported that glucose and amino acid uptake was reduced in sporulating B. subtilis cultures, and that uptake of RNA precursors was reduced by the stringent response to amino acid starvation. Phage infection does not induce either sporulation or the stringent response; however, it is possible that SP01 infection could induce a specific set of physiological responses which might include reduced rates of nucleic acid precursor uptake. For example, overall consumption of nucleic acid precursors could decrease upon phage infection, and through a feedback mechanism subsequently reduce precursor uptake. The results shown in Figure 7 and Table III suggest that the reduced rate of uridine transport in the phage infected cultures had no effect, since the measured rRNA synthesis continued at an undiminished rate after phage infection.

In experiments which investigated the effect of growth rate on burst size it was shown that the burst size of SP82 (an HMU containing DNA phage closely related to SP01) increased logarithmically with host growth rate (Figure 3). Previous work (Hiatt and Whiteley, 1978; Downard and Whiteley, 1981) suggested that SP82 development was regulated through the production of mRNA. As a result of transcriptional regulation, one

might predict that phage production would be limited by either translation or transcription capacity. Lawrie, et al. (1978) estimated the protein coded by SP82 genome as 3×10^6 daltons, which for a burst size of 1200 corresponds to approximately 6×10^{-15} g phage protein per cell. At high growth rates this value is less than 1/10 the amount of protein in a Bacillus cell (E. Leduc and G. Spiegelman, unpublished observations reported in Webb, et al., 1982). Since the synthesis of host mRNA ceases after infection with HMU phages (Hemphill and Whiteley, 1975), there is ample translation capacity for phage proteins.

Table II compares the exponential rate constants for phage burst size, total RNA per cell, and percent active RNA polymerase. The difference in the constants for SP82 burst size and percent active RNA polymerase is not statistically significant, in other words, the SP82 burst size increased at the same rate with growth rate as the percent active polymerase. The exponential rate constant for total RNA per cell, however, differed statistically from the constants of the other two parameters. These data suggested that phage burst size is dependent on active RNA polymerase.

C. RNA synthesis in phage infected B. subtilis

The apparent dependence of HMU phage burst size on the pool of active RNA polymerase provided the original rationale for the experiments reported in Figure 7 and Table III: if RNA polymerase was limiting in the cells at the time of phage infection, then modification of a considerable portion of the polymerase could prevent distribution of more polymerase to the rRNA promoters. Since it is unlikely that SP01-

modified polymerase could be used to transcribe rRNA (see below), the phage induced modification should deplete the polymerase pool and prevent the shift-up response. The data presented in Figure 7 and Table III showed that the percent rRNA synthesis did not increase after a nutritional shift-up in phage infected cells. The control experiment with the uninfected cells showed that the conditions of growth, labeling and shift-up induction were sufficient to induce and detect increased rRNA synthesis. Phage infection appeared to block or alter a chemical reaction or a host gene product which was required to stimulate increased rRNA synthesis.

If phage burst size is dependent on available active polymerase, why isn't phage messenger RNA the only transcription product? Gage and Geiduschek (1971b) reported that host mRNA synthesis drops by 95% in the first minute of infection. Why does the synthesis of ribosomal RNA continue? In vitro experiments strongly suggest that if total replacement of the host sigma⁴³ subunit of RNA polymerase with the phage-coded gp28 subunit occurred, rRNA transcription would stop. The modified polymerase is highly selective for HMU DNA (Duffy and Geiduschek, 1975; Lee, et al., 1980). Furthermore, even if the B. subtilis chromosomal DNA contained HMU, the nucleotide sequences around the start of the 16S gene show no homologies to promoters for gp28 containing polymerase (Talkington and Pero, 1979; Stewart and Bott, 1983). Chelm, et al. (1981) showed that gp28 is more effective at binding to core than is sigma⁴³. Given that the cells were infected at an moi=25, which should theoretically give an abundance of gp28, one would expect that in the phage infected cell most of the polymerase would be complexed with gp28 and not sigma⁴³. However, rRNA synthesis did continue in infected cells,

which implies that there is some polymerase which is inaccessible to gp28 modification. This possibility has also been suggested by Hemphill and Whiteley (1975).

The data presented in Figure 7 and Table III suggest that the RNA polymerase which transcribes rRNA genes is resistant to gp28 modification, and that rRNA specific polymerase is functionally, at least, a stable entity. Two models for the partitioning of RNA polymerase have been suggested by a number of groups working on E. coli. The first model postulates the existence of a protein factor which associates with either RNA polymerase core or holoenzyme such that stable RNA operons are specifically transcribed (Muto, 1978, 1981; Oostra, et al., 1980, Williams, et al., 1983). If this model were translated into the B. subtilis system, polymerase complexed with such a factor might be less effective at binding gp28 and thus allow rRNA transcription to continue. The second model postulates that RNA polymerase holoenzyme exists in two forms modulated by the nucleotide ppGpp: form I specifically transcribes stable RNA operons and form II transcribes all other operons (Travers, 1976; Ryals, et al., 1982). Again, if this model were translated into the B. subtilis system, the form I enzyme would be less effective at binding gp28 and rRNA transcription would continue. Although the data presented in Figure 7 and Table III did not support or contradict either model, the models suggested two different approaches for further characterization of the putative rRNA specific RNA polymerase.

D. Isolation of rRNA specific RNA polymerase

To isolate an rRNA specific RNA polymerase from preparations of enzyme, one of two purification steps was used in conjunction with the standard purification procedure of Dobinson and Spiegelman (1985): affinity chromatography through a pHD1.8 DNA-cellulose column (Figure 10), or sedimentation through a 15-30% glycerol gradient (Figure 11). The activity profile of the polymerase preparation which had been chromatographed through a pHD1.8 DNA-cellulose column showed that the peak of specific transcription was the same for both the rRNA and non-rRNA promoters, but differed from the peak of total activity (Figure 10). Similarly, the activity profile of the polymerase preparation sedimented through a glycerol gradient showed that the peak of specific transcription was the same for both the rRNA and non-rRNA promoters (Figure 11). The assay for total activity measured the RNA synthesized by both core (α_2 , β , β') and holoenzyme (core + σ^{43}), whereas the assay for specific transcripts measured the RNA synthesized by only holoenzyme. The different elution patterns of total activity and specific activity may reflect the different affinities of core and holoenzyme for sites on the DNA cellulose.

Variations of both these methods have been used by others to isolate specific proteins. For example, Pirrotta and Ptashne (1969) used specific DNA fragments complexed to cellulose to isolate the repressor from bacteriophage 434, and Travers, et al. (1980) claimed functional heterogeneity of E. coli RNA polymerase sedimented through a glycerol gradient. Why wasn't an rRNA specific RNA polymerase isolated in the

experiments illustrated in Figures 10 and 11? There are two possible answers to this question.

On a superficial level the techniques might not have been sufficiently sensitive to isolate an rRNA specific polymerase. In the affinity chromatography experiment, the whole plasmid DNA, which contained several E. coli promoters in addition to the B. subtilis rrnB promoters, was complexed to the cellulose rather than a specific promoter fragment. Thus, the ribosomal promoter region sequence was diluted with both other promoters and non-specific sequence. Further, if a DNA fragment containing the tandem promoter had been used, the ratio of non-specific polymerase binding sites to specific binding sites would still have been high, therefore, unless the differential binding to rRNA promoters was very strong, it is not clear whether salt gradient elution would have segregated the putative ribosomal specific polymerase. In the sedimentation experiment the fractions collected from the glycerol gradient were 8 to 4 times larger than the fractions collected in the study by Travers, et al. (1980), thus any possible polymerase heterogeneity might have been missed with the larger fraction size.

On a more basic level, if one of the current models of rRNA synthesis in E. coli (Cole, et al., 1987; Cashel and Rudd, 1987; Bremer and Dennis, 1987; Lindahl and Zengel, 1986) holds for B. subtilis, the wrong question might have been asked. My search for an rRNA specific RNA polymerase grew out of shift-up experiments and hence the expected response was a stimulation of, or positive effect on, transcription from the rRNA promoters. Other studies of specific control of rRNA synthesis centered on the stringent response and the effect of the nucleotide ppGpp

(Glaser, et al., 1983; Sarmientos, et al., 1983; Kingston and Chamberlin, 1981; Travers, et al., 1980). The focus of these studies was the repression of, or negative effect on, transcription from the rRNA promoters. However, Ryals, et al. (1982) widened the role of ppGpp by extending the proposal of Travers (1976) that stable RNA gene activity in *E. coli* was regulated via a ppGpp partitioning of the cellular complement of RNA polymerase into two forms, one with a high and the other with a low affinity for stable RNA promoters. Subsequently, Little, et al. (1983) demonstrated that the rate of stable RNA synthesis as a fraction of the instantaneous rate of total RNA synthesis (r_s/r_t) had an inverse relationship to ppGpp concentration. Theoretically then, when no ppGpp is present, r_s/r_t should equal 1 and all RNA polymerase molecules should be in form I, with a high affinity for rRNA promoters. The polymerase purification procedure may have removed nucleotides and therefore, unless ppGpp is very tightly bound to polymerase, the purified RNA polymerase would theoretically be in form I. In other words, the purified polymerase was already in the rRNA specific form, and further stimulation of transcription from the rRNA promoters would not have been observed. The results of the kinetic analysis of transcription initiation at the rRNA promoters presented in Figure 21 suggested that purified polymerase did have a high affinity for rRNA promoters (see below).

E. Single round transcription assay

Since the current models for the regulation of ribosome synthesis in *E. coli* postulate the initiation of rRNA synthesis as a primary site of control (Jinks-Robertson and Nomura, 1987; Bremer and Dennis, 1987), the logical step in the investigation of rRNA synthesis in *B. subtilis*

was a study of transcription initiation from the rrnB promoters. To focus on a single initiation event rather than multiple rounds of transcription in in vitro assays, the competitive inhibitor heparin, an analogue of single stranded DNA, is included in the productive assay described for E. coli systems (Stefano and Gralla, 1980) and in the single round transcription assay for B. subtilis systems (Dobinson and Spiegelman, 1985).

On all templates the level of RNA polymerase activity was less than 1 transcript per promoter (Figures 12 and 13). Similar levels of polymerase activity at the G2 and A2 promoters have been reported (Dobinson and Spiegelman, 1987). Comparison of transcription levels from different promoters studied in different laboratories has been difficult for a number of reasons. In some cases transcription assays do not include heparin, hence reported transcription levels measure multiple rounds of transcription initiation (for example, Glaser, et al., 1983). Other reports of assays which include heparin do not express transcription activity as transcripts formed per promoter present, but rather as abortive products formed (McClure, 1980) or in relative terms such as counts per minute (Stefano and Gralla, 1982) or as ratios (Petho, et al., 1986).

Several explanations for attaining less than one transcript per promoter in in vitro assays have been proposed. For example, interference by inactive, or excess polymerase, has been suggested (Prosen and Cech, 1985). The significance of this effect has been questioned (D. Straney and Crothers, 1987) and there has been no indication of such inhibition in the work with phage promoters (K.

Dobinson, personal communication) or with the present data. A second reason for low levels of transcription could be that even in the presence of nucleotides, B. subtilis RNA polymerase does not reach a heparin resistant state very rapidly. The levels of transcription observed would reflect the competition between heparin inactivation and elongation. If inactivation is still a rapid event, one would not expect to reach high levels of transcription even when all promoters were complexed with polymerase.

A number of components of the in vitro assay might also lead to the low level of polymerase activity. For example, a linear template rather than a supercoiled template might contribute to low levels of transcription. In E. coli systems it has been well established that in vitro transcription from rRNA promoters is sensitive to the topological state of the template (Petho, et al., 1986; Glaser, et al., 1983). On templates containing E. coli rRNA promoters in transcription reactions without heparin, Glaser, et al. (1983) reported that transcription was stimulated 1000-fold when the template was supercoiled rather than linear. Since it is impossible to obtain greater than 1 transcript per promoter in the single round transcription assay used in my studies, the level of 0.2 transcript per promoter could not be increased a thousand-fold. Supercoiling could stimulate, however, the kinetics of the reaction, that is, one or more of the steps in transcription initiation could be affected by the topological state of the template. Recently, Borowiec and Gralla (1987) proposed that supercoiling may play a role in the closed to open complex transition. They suggested that the energy of supercoiling could be stored transiently in a stressed RNA polymerase-promoter complex before being used to melt DNA sequences near the start-

point of transcription. It is possible that a similar mechanism would operate at the B. subtilis rRNA promoters, and could be manifested in the single round transcription assay as an increase in transcripts per promoter, or in an increase in the rate of some step in the initiation pathway.

Transcription initiation at the rRNA promoters exhibited a number of unexpected characteristics. The enzyme concentration curve (Figure 12) and the initiation rate assay (Figure 13) compared in vitro transcription properties of the tandem rRNA promoters to two well characterized ϕ 29 promoters, A2 and G2. Figure 12 showed that the rRNA promoters required more enzyme to reach maximum heparin resistant complex formation than did the phage promoter. At the A2 promoter, maximum activity was reached at an input ratio of enzyme to DNA equal 5 polymerase molecules per promoter, while the rRNA promoters required a ratio of nearly 10 polymerase molecules per promoter. Travers, et al. (1983) suggested that more than one molecule of RNA polymerase is required for transcription at promoters for genes that encode stable RNA. Lamond and Travers (1985) further suggested that RNA polymerase interactions alone could not account for the low level of in vitro transcription from a tRNA promoter, and postulated that additional factors might be present in vivo that stimulate expression from stable RNA genes.

As with other Bacillus promoters, the formation of heparin resistant complexes at the rRNA promoters was found to be sensitive to the nucleotide composition of the initiation buffer (Dobinson and Spiegelman, 1987). The results presented in Figure 13 demonstrated that on the tandem promoter construct, RNA polymerase could form heparin

resistant complexes in the presence of GTP, UTP, and CTP at both P1 and P2. However, when the ribosomal promoters were on separate templates, as in the experiment shown in Figure 14, polymerase formed heparin resistant complexes in the presence of GUC only at P1 (Figure 14a) and in the presence of AGC only at P2 (Figure 14b). Table V shows the DNA sequence of the promoter region of the rrnB operon and the in vivo transcript initiation sites for both promoters (Stewart and Bott, 1983). The results of experiments using the separated templates seem to agree with the in vivo transcription initiation data, while the data from the tandem promoter template appear to contradict them. In the absence of S1 maps, the 5' end of the transcripts produced from the tandem promoter template could not be identified. It is unclear whether the polymerase begins transcription at P2 with the middle A residue (see Table V) under all initiation conditions or whether the enzyme adjusts the initiating nucleotide as conditions change. The different implications of these two possibilities are discussed below.

F. Heparin resistant complex formation at P1 affects heparin resistant complex formation at P2

The results presented in Figure 15 showed that the formation of stable, heparin resistant polymerase-DNA complexes at P2 of the tandem rRNA promoters was dependent on the formation of heparin resistant complexes at P1. These data also indicated that if RNA polymerase did not form heparin resistant complexes at P1, then the heparin resistant complexes formed at P2 decayed. Since my basic assumption is that heparin resistance reflects an initiated complex, I hypothesized that some step in transcription initiation at the P2 promoter of the tandem rRNA

TABLE V

DNA sequences at the rrnB P1 and P2 promoters^a

P1

TAAAAAACTATTTGCAATAAATAAATACAGGTGTTATATTATTAAACGTCGC⁺

P2

AAAAAAGTTGTTGACAAAAAAGAAGCTGAATGTTATATTAGTAAAGCTGCT⁺

Gram positives

AAAAA....TTGACA...A..A...A.T.TG.TATAATAATAT

E. coli

A.....T.TTGACAT..T.....T.TG.TATAAT

-35

-10

^aDNA sequences [from Stewart and Bott (1983)] are compared with the consensus promoter sequences (Hawley and McClure, 1983; and Graves and Rabinowitz, 1986) for promoters recognized by E. coli sigma⁷⁰ and B. subtilis sigma⁴³ RNA polymerases. The -10 and -35 regions and the in vivo initiation sites for the rrnB promoters are underlined and marked with pluses, respectively (Stewart and Bott, 1983).

promoters was altered by the formation of heparin resistant complexes at the P1 promoter.

Given the current understanding of transcription initiation, significant changes in the reaction could occur at either of two rate limiting steps: the isomerization step (the transition from closed to open complex) or the promoter clearance step (the transition from initiated to elongation complex) (McClure, 1985). As I alluded to above, if the start site of the P2 transcript on the tandem promoter template differed under AGC and GUC initiation conditions, the heparin resistant complexes at P1 could mediate the change in stability of the heparin resistant complexes at P2 at different points in the transcription initiation process. To facilitate the analysis, I will first review the data in point form. I will then present two theories and discuss the implications of each theory for the step controlled in P2 transcription initiation by heparin resistant complexes at P1.

Summary of the data:

1. Heparin resistant P2 transcripts are formed with AGC initiation conditions on both the separated and tandem promoter templates (Figures 14b, 15, 16).
2. P2 complexes decay with AGC initiation conditions on both the separated and tandem promoter templates (Figures 15, 16).
3. Heparin resistant P2 transcripts are not formed with GUC initiation conditions on the separated promoter template (Figure 14a).

4. Stable, heparin resistant P2 transcripts are formed with GUC initiation conditions on the tandem promoter template (Figure 15).
5. Heparin resistant P1 transcripts are not formed with AGC initiation conditions on either the separated or tandem promoter templates (Figure 14b, 15a).

It should be emphasized here that the following theories are based on transcription initiation studies performed on a few promoters with E. coli RNA polymerase and models developed from those studies. To date similar studies for B. subtilis transcription initiation have not been carried out and it is not known how well these E. coli models will translate into the Bacillus system.

Theory 1--Under all initiation conditions the P2 transcript always starts with one of the A residues (see Table V). A basic assumption is that changes in the stability of the polymerase-promoter complex to heparin reflect changes in the isomerization step. Support for this assumption is found in the lacUV5 promoter system where the closed complex is heparin sensitive, while the open complex is heparin resistant (Miller and Burgess, 1978). A P2 transcript was not observed with GUC initiation conditions on the separated promoter template, that is, the binary complex had not formed an open complex; however, on the tandem promoter template, a stable heparin resistant complex was formed at P2. Therefore, the heparin resistant complex at P1 must make the enzyme at P2 on the tandem promoter template heparin resistant in the absence of

nucleotides. Theory 1 implies that the P1 mediated change at P2 occurs primarily at the isomerization step.

Under Theory 1, the decay of heparin resistant P2 complexes observed with AGC initiation conditions is interpreted as instability of the complexes while waiting for the fourth nucleotide, and suggests that P2 may be analogous to an E. coli fast start promoter (see below). The formation of stable, heparin resistant complexes observed with GUC initiation conditions at P2 on the tandem promoter template occurs because the P2 binary complex (now heparin resistant due to the presence of a heparin resistant complex at P1) can proceed through the formation of the first phosphodiester bond and elongation without waiting for nucleotides, that is, the formation of ternary complexes takes place after all four nucleotides are present.

The primary argument for Theory 1 is one of consistency, that is, the in vivo and in vitro start sites would be the same. An additional attraction of Theory 1 is that it postulates that the P2 promoter is a fast start promoter, like other strong in vivo promoter such as the T7 A promoters (McClure, 1980).

A potential argument against Theory 1 is that in the GUC initiation reactions, the concentration of the "initiating" nucleotide (A) was low. At the lambda P_R promoter the dissociation constant for the initiating nucleotide has been reported as approximately 50 times higher than the Michaelis constant of the second nucleotide in the formation of abortive transcripts (McClure, et al., 1978). McClure, et al. (1978) rationalized the disparity between the binding affinities of the initiating tri-

phosphates and the subsequent triphosphates by noting that RNA polymerase does not require a primer and that the stacking stabilization must be supplied by the enzyme alone. They suggested that once the weakly bound initiating nucleotide is positioned properly, the second triphosphate can bind to the complex and trap the first. In the GUC initiation reaction in my experiments, the ATP concentration (10 μ M) was necessarily low because it was the labeled nucleotide, while the concentration of the other nucleotides was 400 μ M, that is, the putative initiating triphosphate was present at one-fortieth the concentration of the subsequent triphosphates. Thus, conditions for an A initiation at the P2 promoter were not very favorable.

Theory 2--The P2 transcript can start at different sites within the initiation region of the promoter. On the tandem promoter template with AGC initiation conditions the P2 transcript probably started with the A residue; heparin resistant complexes were not observed at P1 with these conditions. Since a P2 transcript was not observed with GUC initiation conditions on the separated promoter template, the heparin resistant complex at P1 on the tandem promoter template must have facilitated the initiation of the P2 transcript at another residue, probably the G (see Table V), under GUC initiation conditions. Since the postulated change in start site of the heparin resistant complexes at P2 leads to increased stability of the complexes, Theory 2 implies that the P1 mediated change occurs after the formation of the open complex and possibly in the promoter clearance step at P2.

Initiation start site heterogeneity is not an unprecedented phenomenon. Carpousis, et al. (1982) reported that in in vitro tran-

scription initiation reactions, transcripts from the lacUV5 promoter could start at one of four purines within a six nucleotide initiation region. They also found that the relative distribution of transcripts initiated from a single site was dependent on the concentrations of the initiating nucleotides. Further, they concluded that the choice of transcript start sites was dependent on events that occurred after the formation of the open complex and involved the initiating nucleotide. The P2 promoter of the B. subtilis rrnB operon might be amenable to changes in start sites, since the in vivo initiation site for the P2 transcript is located closer to the -10 region of the promoter than most bacterial transcription start sites (Table V; Graves and Rabinowitz, 1986).

The promoter clearance step has been best characterized for the E. coli slow start promoter lacUV5 (Carpousis and Gralla, 1985; D. Straney and Crothers, 1987). Straney and Crothers (1987) proposed a model for promoter clearance in which open complex (polymerase-DNA) contacts compete with initiated complex (polymerase-DNA-RNA) interactions to produce a "stressed intermediate" during the formation of a short RNA-DNA duplex. They further proposed that the strain energy in the ternary complex is relieved by either ejection of the short RNA, which results in aborted initiation, or by elimination of the sigma subunit and breaking of open complex contacts, which results in productive transcription.

Using Theory 2 and the Straney and Crothers model, the decay of heparin resistant complexes at P2 observed with AGC initiation conditions is interpreted as weakened contacts in the -10 region; that is, under normal transcription conditions (no heparin) the the resolution of the

stressed intermediate complex would favor the promoter clearance step. However, under the single round transcription conditions the polymerase did not make strong open complex contacts, therefore, the enzyme came off the DNA. The P1 mediated change in stability of the heparin resistant complexes at the P2 promoter on the tandem promoter template with GUC initiation conditions is thus interpreted as the resolution of the stressed intermediate such that the expulsion of the abortive product and the reversion to the open complex were favored, that is, the open complex contacts at P2 were strengthened by the heparin resistant complexes at P1 and the enzyme stayed on the DNA.

A difficulty with this interpretation of the results is found in the putative sequences of the respective nascent RNAs. Straney and Crothers (1987) postulate that promoters that favor the promoter clearance step may minimize the restraint caused by the competing energies by including mechanisms that strengthen the RNA-DNA duplex. According to this hypothesis, one would expect that the RNA-DNA duplex formed during AGC initiation would have a higher G + C content and therefore be more stable, however, the enzyme could potentially synthesize only a 4-base nascent RNA which would have a G + C content of 50% (see Table V). In contrast, with GUC initiation conditions the enzyme could synthesize an 8-base nascent RNA which would have a G + C content of 62.5%.

At present the data are insufficient to make a choice between Theory 1 and 2. As I noted above these theories are adapted from models based on transcription initiation studies of a limited number of E. coli promoters, it is likely that transcription initiation at B. subtilis

promoters, and even at other E. coli promoters, may not fit into these models.

A mechanism--A mechanism for how heparin resistant complexes at P1 might effect the change in polymerase-promoter interaction at P2 has not been established. However, it is unlikely that the binding of polymerase to P1 is sufficient to mediate the change. The results shown in Figure 16 suggested that when P1 was physically present and available for enzyme binding (the tandem promoter construct, AGC initiation), the heparin-resistant complexes formed at P2 decayed at the same rate as complexes formed on the separated P2 construct (P1 physically absent). In addition, gel retardation experiments demonstrated that the binding of RNA polymerase to P1 did not affect the binding step in P2 initiation (Wakarchuk and Spiegelman, unpublished observations).

A possible mechanism for the effect of P1 on P2 can be suggested if one assumes that alterations in the DNA structure occur when a heparin-resistant complex is formed at P1. DNase I footprints on the veg promoter and the G2 promoter showed that RNA polymerase protected residues -45 to +30 (Le Grice and Sonneshein, 1982; Brion and Spiegelman, in preparation). If the enzyme protects similar regions at both P1 and P2, two RNA polymerase molecules would come within 14 base pairs of one another. DNA unwinding induced by E. coli RNA polymerase has been measured in binary, initiation, and ternary complexes, all of which had an unwinding angle of 17 ± 1 base pairs (Gamper and Hearst, 1982). Ellison, et al. (1987) have demonstrated that a supercoiling-dependent transition occurring in one sequence can act at a distance to affect the behavior of other transitions occurring elsewhere within a given

topological domain. They also note that any change in the energetic requirements of a single sequence, such as protein binding, is likely to affect the pattern of events in all other sequences competing within a given topological domain. Thus, at the rRNA tandem promoters the formation of heparin resistant complexes at P1 could cause alterations in the template structure which could affect the contacts between RNA polymerase and the P2 promoter.

G. Kinetic analysis of transcription initiation at the rRNA promoters

The composite assay used in Section E of In Vitro Results was developed to investigate the rate and mechanism of open complex formation leading to productive transcript formation for a series of mutant E. coli lac promoters (Stefano and Gralla, 1982). This assay has also been used, with some modifications, to determine the rate of heparin resistant complex formation for the B. subtilis ϕ 29 A2 promoter (Figure 19a; Dobinson and Spiegelman, 1985).

Three kinetic parameters for polymerase-promoter interactions can be derived from tau plots: (1) the overall forward rate constant for the formation of initiation complexes, K_{on} , from the slope of the line; (2) the equilibrium dissociation constant, K_A^* , from the x-intercept; and (3) the rate constant for the conversion of the unstable intermediate to the initiated complex, k_2 , from the y-intercept. The tau plots of polymerase-promoter interactions for the lac promoters and the A2 promoter resulted in lines with a positive slope (Stefano and Gralla, 1982; Figure 19a; Dobinson and Spiegelman, 1985), thus, permitting the calculation of all three parameters. The tau plot for the tandem rRNA

promoters, however, resulted in lines with flat or slightly negative slopes (Figure 21), hence, K_{on} and K_A^* could not be determined using this method.

What are the implications of a shallow slope on a tau plot for polymerase-promoter interactions? K_A^* has also been referred to as K_B , the equilibrium binding constant of polymerase to promoter (McClure, 1985). Using this latter designation, the results presented in Figure 21 suggest that a polymerase molecule is always bound at the rRNA promoters. A shallow positive or a flat slope in a tau plot is not unprecedented. A tau plot analysis of phage T7 A1, A2, and A3 promoters (derived from the abortive transcript assays) resulted in lines with shallow or flat slopes for those three strong promoters (McClure, 1980; Dayton, et al., 1984). McClure (1980) predicted that frequently initiating promoters as a class would have low y-intercepts and low slopes. The y-intercepts for both the rRNA promoters were higher than any determined for the T7 promoters, the lacUV5 promoter or the ϕ 29 A2 promoter.

The major weakness in using either the abortive initiation or the composite assays to obtain kinetic parameters is that both are indirect and might not reflect all the intermediate steps in the formation of an initiated complex. In addition, they require a number of assumptions to be made in order to derive kinetic values (Stefano and Gralla, 1982). The abortive initiation assay, however, is ideally suited to investigate the dynamics of the promoter clearance step (see above). A gel retardation method, which measures initiation products directly, has been used to determine the kinetics of transcription initiation at the lacUV5 promoter (Straney and Crothers, 1987). The gel retardation method, as

described, requires a DNA fragment containing a single promoter, and as demonstrated above (Figures 15-18) stable transcription initiation from the P2 promoter is dependent on the presence of a stably initiated complex at P1, in other words, two promoters on a DNA fragment. It is possible that the gel retardation method will be useful in establishing the kinetic parameters for the B. subtilis rRNA tandem promoters, if a clear characterization of all potential bands on a gel can be made.

H. Transcription initiation from promoters on artificial tandem promoter templates

The data presented in Figures 14, 15, and 16 indicated that in order for RNA polymerase to form stable, heparin resistant complexes at the rrnB P2 promoter, heparin resistant complexes must also be formed at the rrnB P1 promoter. The investigations of transcription initiation at promoters on artificial tandem promoter constructs were designed to limit possible mechanisms for the P1 mediated effect on the formation of heparin resistant complexes at the P2 promoter.

Transcription initiation on pPldP2--In both B. subtilis and E. coli the tandem promoters of the ribosomal operons are separated by about the same distance, 90 to 110 base pairs (K. Bott, personal communication; Lindahl and Zengel, 1986). Is this distance between the promoters critical?

Experiments using plasmid pPldP2 set out to examine the effect on transcription initiation of increasing the distance between the rrnB promoters from 90 to 185 bases. Since the rate and final level of tran-

scription initiation at the P1 promoter was unaffected by the presence or absence of the P2 promoter (Figure 17), the initial prediction was that transcription initiation from P2 might be depressed by increasing the distance between the tandem promoters, and that the pattern of transcription from P1 would be unaffected. The results presented in Figure 23 and Table IV, however, indicated that transcription initiation at both rRNA promoters was reduced on the pPldP2 template. When the polymerase concentration was increased, the levels of transcription at both promoters on pPldP2 approached those observed on the wild type template (Table IV). However, it is unlikely that a concentration effect could provide a simple explanation for these observations, since the polymerase to promoter ratio was higher in reactions in which pPldP2 DNA was the template, than in reactions in which pHD1.8 DNA was template.

The interpretation of these data is further complicated by the existence of promoter-like sequences between P1 and P2 created in the cloning process of the pPldP2 template (Figure 22). As noted above, Ellison, et al. (1987) have postulated that supercoiling dependent transitions occurring in one sequence can act at a distance to affect the behavior of other transitions occurring elsewhere within a topological domain. They also suggest that any change in the energetic requirements of a single sequence through mutation, base modification or protein binding is likely to affect the pattern of events in all other sequences competing within the domain. It is possible that the promoter-like sequence between the two rRNA promoters competed with the two true promoters and therefore changed the energetic requirements for both P1 and P2 on the pPldP2 template.

Transcription initiation on pPlA2--The formation of heparin resistant complexes at P1 on the tandem promoter template permitted the formation of stable, heparin resistant complexes at the P2 promoter (Figures 14, 15, and 16). Was this P1 mediated effect limited to the P2 promoter or would P1 affect transcription initiation at other promoters?

The aim of the experiments using the plasmid pPlA2 as a template was to investigate the effect of the rrnB P1 promoter on transcription initiation at the ϕ 29 A2 promoter. Theory 2 (see above) postulated that the formation of heparin resistant complexes at P1 enabled the transcript formed at P2 to initiate with a GTP residue, an event which did not occur on the separated P2 promoter template (Figure 14a). While the results presented in Figure 25 demonstrated that the presence of the P1 promoter upstream of the A2 promoter did not alter the initiation nucleotide requirements for the formation of heparin resistant complexes at the A2 promoter, they did suggest that the presence of P1 stimulated transcription activity at the downstream promoter.

Results from subsequent experiments (Figures 26 and 27) suggested that when the P1 promoter was physically linked to the A2 promoter and initiation conditions permitted, transcription initiation was stimulated from the A2 promoter. Unlike the situation at the P2 promoter, a heparin resistant complex at the P1 promoter was not required for an effect at the A2 promoter. Under these conditions the P1 promoter might be acting like the activator regions reported upstream of many natural promoters in both E. coli and B. subtilis (Gourse, et al., 1986; Lamond and Travers, 1983; Banner, et al., 1983; Horn and Wells, 1981). The kinetic properties of the P1 promoter, high affinity for the polymerase and slow

initiation rate (Figure 21), would support the role of polymerase "feeder" to a downstream promoter.

I. Summary

I began this investigation with a study of rRNA synthesis in B. subtilis during steady state growth and under nutritional shift-up conditions. I demonstrated that in B. subtilis the fractional rate of rRNA synthesis increased as a function of growth rate, and was similar to that reported for E. coli. The kinetics of rRNA synthesis after a nutritional shift-up in B. subtilis, however, did not conform to those established for E. coli. I also examined the relationship between rRNA synthesis and RNA polymerase availability using an amber mutant of the SP01 phage and found evidence which suggested the existence of a ribosomal RNA specific RNA polymerase.

The conclusions from the in vivo study led to an analysis of rRNA transcription in vitro. When I undertook the isolation of the putative ribosomal RNA specific RNA polymerase, I observed no difference in activity profile when transcription activity at the rRNA tandem promoters was compared to activity at a non-ribosomal promoter. In vivo analysis of the control of rRNA synthesis in E. coli suggested that regulation occurred on the level of transcription initiation, therefore, I turned my in vitro investigation to transcription initiation at the B. subtilis rRNA promoters using the single round transcription assay. I showed that the formation of a heparin resistant complex at the P1 promoter affected the stability of the heparin resistant complex formed at the P2 promoter. I examined the kinetics of transcription initiation at the rRNA promoters

and demonstrated that RNA polymerase had a high affinity for both rRNA promoters, but the rate of initiation at these promoters was relatively slow when compared to non-ribosomal promoters. Finally, I compared transcription initiation on two artificial tandem promoter constructs with initiation on the native tandem promoter construct, and found that while the presence of the P1 promoter had specific effects when it was upstream from the different promoters, in general, P1 had a positive effect on transcription from downstream promoters.

The work described in this thesis contributes to the understanding of the synthesis of rRNA in the following areas. My in vivo investigation showed that in terms of global cellular regulation the synthesis of rRNA in B. subtilis is similar to that observed in E. coli. Since I was unable to isolate a rRNA specific RNA polymerase, I cannot offer a precise mechanism for growth rate regulation at the rRNA promoters. However, my investigation of transcription initiation did suggest some mechanisms for regulation at the level of initiation. While transcription initiation has been studied in vitro at many E. coli promoters including some tRNA promoters, no tandem rRNA promoters have been examined in this way. My investigation of transcription initiation at the tandem promoters of B. subtilis indicated that the rate and final level of formation of heparin resistant complexes was higher at P2 than at P1, and supported the in vivo observation that the P2 promoter of the B. subtilis rrnB operon when expressed in E. coli was the transcriptionally more active of the tandem rRNA promoters (Deneer and Spiegelman, 1987). The results from initial rate assays at native and artificial tandem constructs suggested that the B. subtilis P1 promoter might act like the stimulatory elements found upstream of the stable RNA

promoters in E. coli (Gourse, et al., 1986; Lamond and Travers, 1983; Banner, et al., 1983; Horn and Wells, 1981). I proposed that the heparin resistant complexes formed at the P1 promoter changed the polymerase-DNA contacts at the P2 promoter and thereby altered either the isomerization or the promoter clearance step in transcription initiation at the P2 promoter, by modifying supercoiling-dependent transitions within a given topological domain. Finally, the tau plot kinetic analysis of the tandem rRNA promoters showed that the kinetics of transcription initiation from these promoters were characteristic of other promoters which are very active in vivo, namely the phage T7 A promoters (McClure, 1980).

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