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Promoter and nonspecific DNA binding by the T7 RNA polymerase

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ABSTRACT

T7 RNA polymerase plays an important role in both the transcription and replication of bacteriophage T7. In this study we have used a nitrocellulose filter binding assay to examine the binding properties of the T7 RNA polymerase with T7 promoters cloned into plasmid DNAs. Promoter-specific binding was shown to be relatively insensitive to variations in the ionic strength of the incubation solution but dependent on the helical structure of the DNA. On the other hand, nonpromoter interior-site binding was independent of the superhelicity of the DNA but extremely sensitive to changes in the ionic strength. These results suggest that nonspecific binding results from ionic interactions between positively charged residues of the polymerase and the polyanionic backbone of the DNA, whereas promoter-specific binding is dependent upon base-specific contacts within the promoter sequence. A comparison between the transcriptional activity and binding strengths of the RNA polymerase to specific promoters indicates little correlation between these two properties. This suggests that differential promoter binding does not represent a major mechanism for regulating transcription in bacteriophage T7. Instead, factors which influence the efficiency or rate of formation of the polymerase-promoter open complex are found to have the major role in determining transcriptional levels in this system.

INTRODUCTION

Bacteriophage T7 is a lytic phage having a linear duplex chromosome which contains 39,936 base pairs. In addition to being well characterized genetically, the complete nucleotide sequence of the T7 DNA molecule has been published (1). Following infection of *E. coli*, phage T7 produces at least 38 proteins during its life cycle. Genetic and biochemical analysis has revealed that functionally related genes are clustered along the chromosome (2, 3) and that the production of the various T7 gene products are temporally regulated (2). The early or class I genes are transcribed by the *E. coli* RNA polymerase and are the first to appear following infection, while the class II and class III genes are transcribed later by the T7 RNA polymerase—the product of the gene 1 (2).

In addition to the *E. coli* RNA polymerase promoters responsible for class I RNA transcription, the T7 genome contains 17 promoters for the T7 RNA polymerase (1). These include five class III promoters, all of which possess the same 23 base pair sequence, ten class II promoters, and two so called replication promoters, ϕ0L and

ϕ0R. The sequences of the class II and ϕ0L promoters differ from the 23 base pair class III sequence by from one to several nucleotides. The replication promoters apparently lie near secondary origins of replication (4, 5) and may possibly be used in later stages of T7 DNA replication (5). Two class II promoters are directly adjacent to the primary origin of T7 DNA replication (6) and have been implicated, along with the T7 RNA polymerase, in the molecular mechanism for initiating the first round of T7 DNA replication (7, 8).

Transcription from class II and III promoters has been well characterized both *in vivo* and *in vitro* using either intact T7 DNA or plasmids containing cloned T7 promoters (9). Although class II promoters are the first to be utilized following phage infection (10) no temporal differences between the initiation of class II and III transcription is observed after T7 infection if the promoters are located on plasmids contained within the infected *E. coli* cell (11). Since the left genetic end of the T7 DNA, which contains the class II promoters, enters the cell first, it has been suggested that this phased entry of the infecting T7 DNA may account for the temporal initiation of gene expression (11). It is not clear, however, how class III gene transcription is preferentially maintained later in the phage T7 life cycle although *in vitro* studies have shown that class III promoters are, in general, stronger than class II promoters (9) and are less sensitive to high ionic strength (10). Presumably the minor sequence differences between class II and III promoters are responsible for the differences in these physical properties.

In this paper we have used nitrocellulose filter binding assays to examine the *in vitro* binding characteristics of the T7 RNA polymerase to both class II and III T7 RNA polymerase promoters cloned into plasmid DNAs. We have also correlated transcription and binding as a function of both the helical structure of the DNA and the ionic strength of the binding reaction and conclude that transcription is not regulated in the T7 system by a mechanism of differential promoter binding.

MATERIALS AND METHODS

Phage and Bacterial Strains

T7 *amber* mutants were obtained from F. W. Studier (Brookhaven National Laboratories). The *amber* mutants used were: gene 3, *am*29; gene 4, *am*20; gene 5, *am*28; gene 6, *am*147. *E. coli* 011' *su*$^+$ *thy* (12) and *E. coli* B/1 *su*$^-$ *T1*^{r} (13) have been previously described. *E. coli* RR1 containing the plasmid pBR322 and *E. coli* HMS174 were obtained from C. Richardson (Harvard University). Plasmid pAR111 (T7*14.1-18.2*) was obtained from F. W. Studier. Plasmid pDR100 (T7*67.5-74.1*) has been described (7).

DNA and Nucleotides

Unlabeled and [^{3}H]-labeled plasmid DNA was isolated by a minor modification of a

previously described method (14). Supercoiled and nicked circular DNA was separated by centrifugation to equilibrium in CsCl (14). Closed circular relaxed plasmid was prepared by ligation of the nicked circular plasmid DNA with T4 DNA ligase. Complete ligation was demonstrated by agarose gel electrophoresis in the presence of ethidium bromide and by the inability of the ligase-treated DNA to support nick translation by DNA polymerase I. Unlabeled nucleotides were obtained from P-L Biochemicals. Labeled nucleotides were from ICN Pharmaceuticals.

Preparation of Recombinant Plasmids Containing T7 Promoters

The 1778 base pair *Bam* HI fragment from pAR111, which contains the T7 ϕ1.1A, ϕ1.1B, and ϕ1.3 promoters, and the 2625 base pair *Pst* I fragment from pDR100, which contains the T7 ϕ13 promoter, were purified by electroelution from agarose gels followed by chromatography on DEAE cellulose (14). The purified *Bam* HI and *Pst* I fragments were subsequently digested with *Hpa* II/*Taq* I or *Hpa* II, respectively, and ligated into the *Cla* I site of pBR322. Individual bacterial colonies were selected from *E.coli* HMS174 transformed cells which had been enriched for tetracycline-sensitive cells by treatment with cycloserine (15). DNA isolated from these colonies was determined to contain T7 promoters based on its ability to serve as a template for transcription by the T7 RNA polymerase. The nature and orientation of the promoter inserts was determined by DNA sequencing (16). The clones used in this study contained either the T7 ϕ1.1B (pSS104), or ϕ13 (pSS110) promoters.

Enzymes

T7 RNA polymerase (specific activity 180,000 units/mg) was purified from $T7_{3,4,5,6}$-infected *E. coli* B/1 by the method of Fischer and Hinkle (17). Restriction enzymes, *E. coli* DNA polymerase large fragment, and T4 DNA ligase were from New England Biolabs.

Binding Reactions

The binding of the T7 RNA polymerase to DNA was measured in a solution (final volume 0.2 ml) containing 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 50μg bovine serum albumin, and 1 nmol [^{3}H]-labeled plasmid DNA (2,000-4,000 cpm/nmol). T7 RNA polymerase and NaCl were added as indicated. Reactions were incubated for 10 minutes at 37 °C, diluted, and filtered onto 25 mm nitrocellulose disks (Millipore #HAWP 02500) as described by Hinkle and Chamberlin (18). Filter-bound radioactivity was determined by counting in a Beckman 7500 liquid scintillation counter using a toluene-based fluor. Restriction enzymes, polymerase I, and DNA ligase were removed prior to the binding reaction by phenol extraction followed by precipitation of the DNA with ethanol. Competitive binding reactions were carried out as described above except that reactions contained 5μg each of unlabeled pSS104, pSS110,and pBR322 DNA and 50 units of T7 RNA polymerase.

NaCl was added as indicated. Following filtration, the DNA was recovered from the filter (19),and digested with *Rsa*I. Following electrophoresis on agarose gels, the relative levels of DNA in each band was determined by scanning a negative photographic image of the gel using a Hoefer GS300 densitometer and a Hewlett Packard 3380A integrator. Control lanes containing known amounts of digested plasmid were included with each electrophoretic run.

Transcription

RNA synthesis by the T7 RNA polymerase (50 units) was measured in a reaction (final volume 0.05 ml) containing 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 12.5µg bovine serum albumin, 0.4 mM rNTPs, and 1 nmol of either *Dde*I (pSS104) or *Rsa*I (pSS110) digested DNA as indicated. The radioactive label was included at a concentration of 0.2 mM as [γ-^{32}P] GTP (8,000-12,000 cpm/pmol). NaCl was added as indicated. Reactions were initiated by the addition of enzyme, incubated for 10 minutes at 37 °C, and the products analyzed by polyacrylamide gel electrophoresis. Competitive synthesis reactions were carried out as described above except that these reactions contained 12.5 units of T7 RNA polymerase, 1.25µg each of pSS104, pSS110, and pBR322 DNA, and the indicated amounts of NaCl. Competitive reactions were preincubated with polymerase for 10 minutes at 37 °C. Synthesis was initiated by the addition of [γ-^{32}P] GTP and continued for an additional 10 minutes prior to analysis of the reaction products by polyacrylamide gel electrophoresis. Following electrophoresis and autoradiography, gel slices corresponding to transcripts were cut from the gels, soaked overnight (18-20 hrs.) in Beckman READY-SOLV MP aqueous scintillation fluid (10ml), and counted in a Beckman 7500 liquid scintillation counter. From control samples containing known amounts of labeled DNA the counting efficiency of this method was determined to be 51% (not shown).

Other Methods

Agarose and denaturing acrylamide/urea gels were prepared and run as described (14). Restriction enzyme digestions were performed as suggested by the supplier. RNA was separated from unincorporated label as described (7).

RESULTS

Retention of Plasmids Containing T7 promoters by T7 RNA Polymerase on Nitrocellulose Filters

To study the promoter and nonspecific (nonpromoter) DNA binding properties of the T7 RNA polymerase we have used a nitrocellulose filter binding assay and plasmids containing cloned T7 promoters. These plasmids included pAR111, which contains the class II ϕ1.1A, ϕ1.1B, and ϕ1.3 promoters, as well as the primary origin

Table I

Retention of Plasmid DNA on Nitrocellulose Filters by the T7 RNA Polymerase[a]

	percent DNA retained	
NaCl (mM)	pAR111	pBR322
0	>95	40
40	60	0
100	40	0

[a]Percentages of supercoiled pAR111 and pBR322 bound to nitrocellulose filters were determined as described in "Materials and Methods" and contained 150 units of T7 RNA polymerase and 1nmol of the indicated DNA.

of T7 DNA replication; pSS104, which contains the ϕ1.1B promoter and the primary replication origin; and pSS110, which contains the class III ϕ13 promoter.

Using these plasmids, we have found that T7 RNA polymerase was able to effectively retain promoter-containing DNA on nitrocellulose. For example, at high T7 RNA polymerase concentrations (150 units) essentially 100% of the plasmid pAR111 was retained if the binding reaction was carried out at low ionic strengths (Table I). However, a portion of this binding must represent nonpromoter interactions since under comparable conditions, 40% of the pBR322 control DNA was retained (Table I). Even at low ionic strengths, this binding was dependent on the RNA polymerase since in its absence or in the presence of equivalent amounts of BSA, no (<5%) binding was observed with promoter or nonpromoter-containing DNA. Furthermore, when the NaCl concentration in the binding reaction exceeded 40 mM, the nonpromoter binding was no longer detectable, although under these conditions the promoter-dependent retention was also reduced (Table I).

Retention of Nonpromoter T7 RNA Polymerase Complexes on Nitrocellulose Filters

At low ionic strengths nonspecific (nonpromoter) polymerase-DNA complexes resulted in substantial retention of DNA on a nitrocellulose filter (Table I). Therefore, prior to studying promoter-polymerase interactions, it was necessary to determine the contributions of these nonspecific interactions on the binding curves obtained using promoter-containing plasmids. In addition, once the extent of binding as a function of ionic strength was known, it was possible to carry out promoter binding studies at ionic strengths where the nonpromoter contributions were negligible. Shown in Fig. 1A is the effect of increasing ionic strength on nonpromoter binding between the T7 RNA polymerase and either supercoiled or linear pBR322 DNA. Both

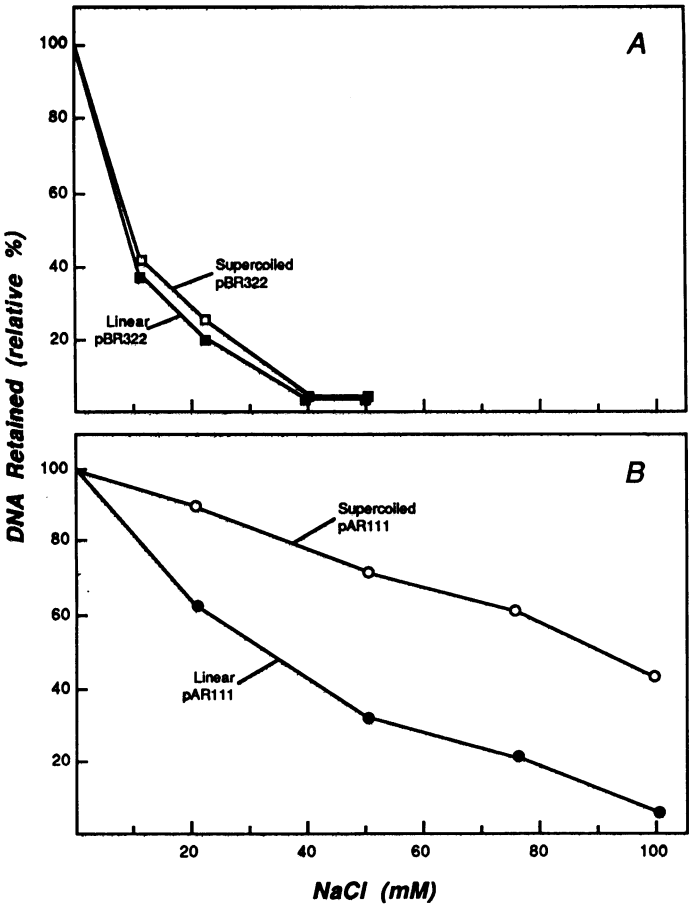

Fig. 1. Relative binding of T7 RNA polymerase to supercoiled and linear pBR322 and pAR111. Binding assays were carried out as described in "Materials and Methods" and contained 50 units of T7 RNA polymerase and 1 nmol of either the supercoiled or linear (*Pvu*II or *Eco*RI linearized) form of the indicated [^{3}H] plasmid DNA. 100% values: pBR322, supercoiled, 0.10 nmol, linear, 0.10 nmol ; pAR111, supercoiled, .75 nmol, linear, 0.27 nmol.

forms of DNA bound the T7 RNA polymerase to a similar extent and in both cases this binding exhibited identical sensitivities to increased ionic strength. When NaCl was present at concentrations in excess of 40 mM, nonpromoter binding to either form of pBR322 DNA was undetectable (Fig. 1A).

Retention of the promoter-containing plasmid pAR111 was also dependent on ionic strength but exhibited much less sensitivity than that observed with pBR322.

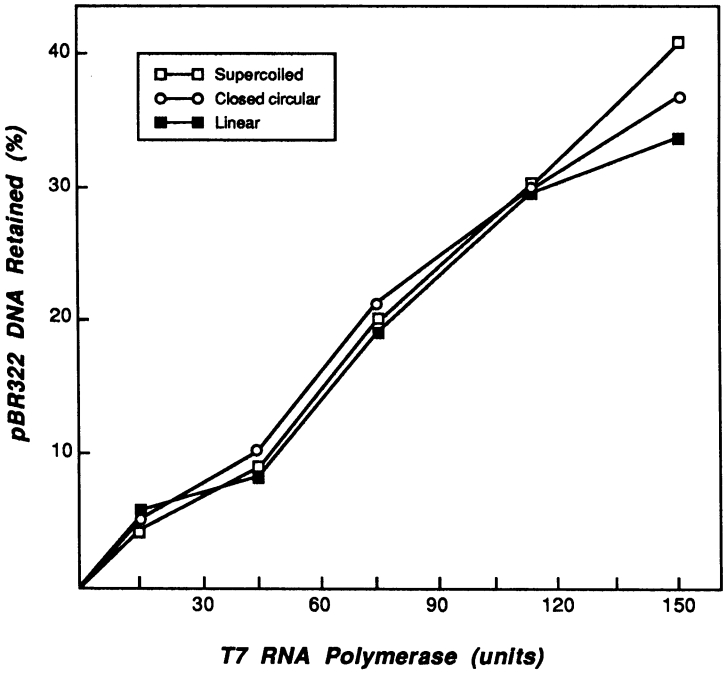

Fig. 2. Nonspecific binding of T7 RNA polymerase to supercoiled, linear, or closed circular pBR322 DNA. Binding reactions were carried out in the absence of NaCl as described in the "Materials and Methods" section using the indicated amount of T7 RNA polymerase.

Furthermore, the supercoiled and linear forms of pAR111 were bound to differing relative extents at each of the ionic strengths tested (Fig. 1B). Thus, in the presence of 50 mM NaCl when nonspecific binding was negligible, 70 and 30% of the respective maximum amounts of the supercoiled and linear forms of pAR111 DNA was retained by the T7 RNA polymerase. Unlike nonpromoter binding, which was not influenced by the topological state of the DNA, the interaction between the T7 RNA polymerase and the promoter was strongly dependent on the superhelical density of the DNA. We found that supercoiled promoter-containing DNA was more effectively bound by the polymerase under all experimental conditions (Fig. 1B, and below).

We have further characterized the nonpromoter interactions between the T7 RNA polymerase and DNA by comparing the retention of the supercoiled, linear, and closed circular relaxed forms of pBR322. These results, presented in Fig. 2, show essentially no difference in the ability of the polymerase to bind to the various forms of this plasmid, suggesting that nonspecific binding is independent of the topological

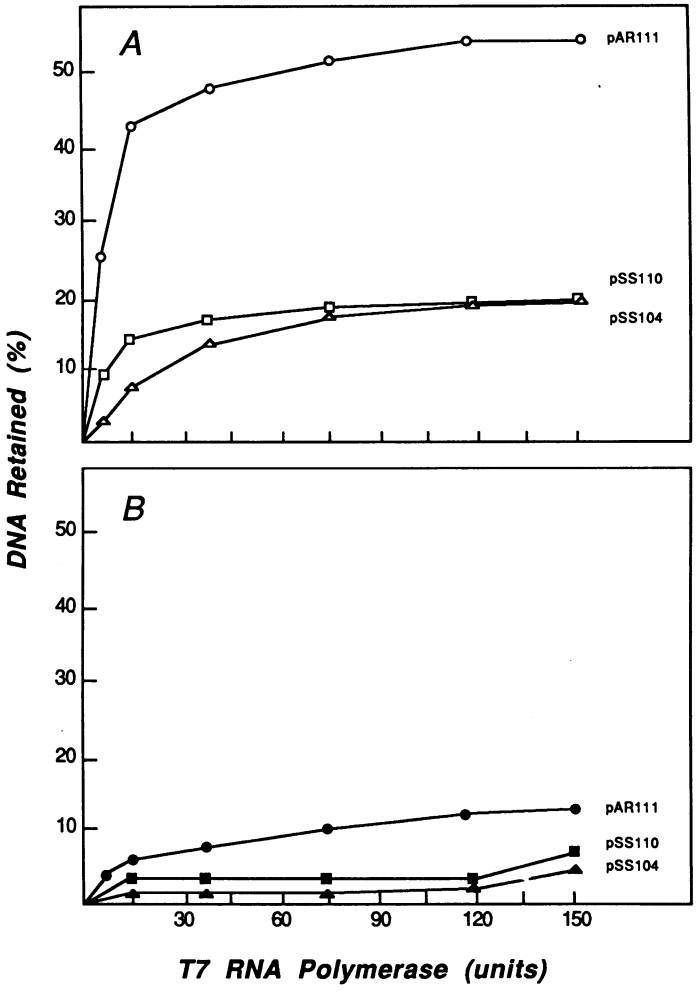

Fig. 3. Promoter specific binding of T7 RNA polymerase. Supercoiled or linear forms of pAR111, pSS104, or pSS110 DNA was incubated with the indicated amounts of T7 RNA polymerase in the presence of 50 mM NaCl and the binding measured as described in "Materials and Methods." Under these conditions, binding to pBR322 is not detectable.

state of the DNA. Also, the fact that circular and linear DNAs were equally retained suggests that interactions between the RNA polymerase and the termini of the DNA molecules did not contribute to the binding.

Promoter-Specific Binding by the T7 RNA Polymerase

We have measured the extent of promoter-specific binding as a function of T7

RNA polymerase concentration using both supercoiled and linear forms of pAR111, pSS104 and pSS110 DNA under ionic conditions (50 mM NaCl) where nonspecific binding was negligible (Fig. 3). Retention of promoter-containing DNA under these conditions was strongly influenced by the topological state of the DNA. Using supercoiled DNA, a maximum of 60% of the pAR111 and 20% of the pSS104 and pSS110 DNA was retained at high RNA polymerase concentrations (>100 units). At this level (100 units) there are approximately 30 RNA polymerase molecules present per DNA molecule. However, we have not determined the fraction of RNA polymerase molecules that are active nor is it known whether molecules inactive in the polymerization reaction can still bind and retain DNA on a nitrocellulose filter.

Upon linearization, retention of all promoter DNA was substantially reduced. In fact, with the plasmids pSS104 and pSS110, no retention was observed except at extremely high RNA polymerase concentrations. The possibility that these binding differences reflect an inherent ability of the filter binding assay to differentially retain supercoiled and linear forms of DNA is discounted by the data obtained for nonspecific binding. In this case both the linear and supercoiled forms of pBR322 DNA were equally retained at low ionic strengths.

While these studies indicate that polymerase-promoter specific complexes may be effectively retained on nitrocellulose filters, Fig. 3 shows that the efficiency of this retention is relatively low. That is, at 1:1 molar ratios of polymerase and promoter, less then 10% of the DNA is retained on the filter. This result, which is in contrast to efficiency values of 40-80% that have been reported for the *E.coli* RNA polymerase (18), has been observed previously for the T7 RNA polymerase and reflects the relative weakness of the polymerase-promoter interaction (20).

Relationship Between Transcription and Binding as a Function of Ionic Strength

To compare the salt and class-dependent levels of promoter-specific binding by the T7 RNA polymerase, we have carried out binding reactions at various ionic strengths using supercoiled plasmids that contained either a single class II (pSS104) or class III (pSS110) T7 RNA polymerase promoter. The contributions from nonspecific binding at low ionic strengths were eliminated by subtracting control values obtained using pBR322. This reduced the relative promoter-specific binding at 0 and 25 mM NaCl by 10% and 2%, respectively. We have also measured the levels of the initiation of transcription under these same conditions using *Rsa*I (pSS110) or *Dde*I (pSS104) digested plasmids as the DNA templates. RNA synthesis from such digests results in run-off transcripts having lengths of 60 and 68 nucleotides for transcription initiated from the ϕ13 and ϕ1.1B promoters, respectively. Synthesis was measured using [γ-^{32}P] GTP which represents the first nucleotide incorporated during transcription from both of these promoters (1).

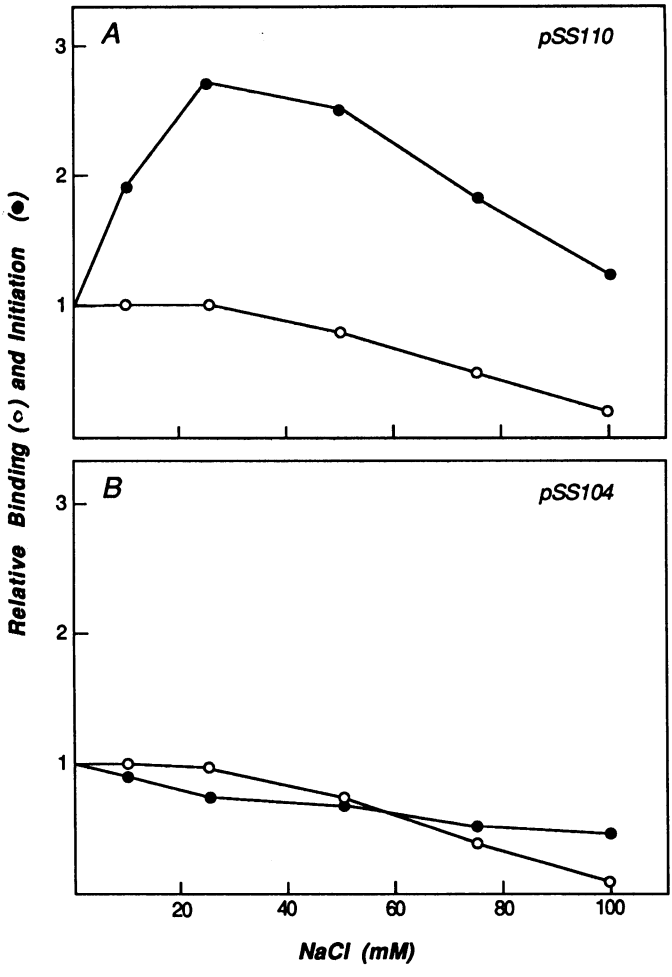

Fig. 4. Relative levels of initiation and binding of T7 RNA polymerase (50 units) to (A) pSS110 and (B) pSS104 DNA templates. Initiation and binding were measured as described in "Materials and Methods" in the presence of the indicated amounts of NaCl. Binding values are corrected for nonspecific binding by subtracting the amount of pBR322 DNA retained at each ionic strength (see text). Values of 100% for initiation (pmol GTP) and binding (corrected nmol retained) for each panel are, respectively: A (pSS110) 0.11, 0.58; B (pSS104) 0.18, 0.55.

The results of these experiments (Fig. 4) indicate that binding by the polymerase to both class II and class III promoters exhibits essentially identical sensitivities to increases in the ionic strength. In contrast, the relative levels of initiation from these promoters differed markedly as the ionic strength was varied. With the class III ϕ13

promoter, initiation was initially stimulated as the ionic strength of the reaction was increased. This was followed by a relative decrease in the levels of synthesis at NaCl concentrations in excess of 25 mM (Fig. 4A). The levels of initiation from the class II ϕ1.1B promoter, on the other hand, continuously decreased as the ionic strength of the reaction was raised (Fig. 4B). Similar results have been observed by others for transcription from both class II and class III promoters (21). A comparison of the levels of promoter binding at various ionic strengths, with the initiation of RNA synthesis, indicates that these properties are not always correlated. This is most apparent for initiation and binding to the class III ϕ13 promoter (Fig. 4A) at low ionic strengths.

To further understand the initiation and binding properties of these promoters, reactions were carried out under competitive conditions using an excess of promoters to polymerase molecules (2.7:1). Binding reactions contained supercoiled pSS104 and pSS110 promoter DNA, as well as nonspecific pBR322 DNA as an internal control. Following filtration, the filter-bound DNA was recovered and digested with *Rsa*I. Since digestion with this restriction endonuclease results in at least one uniquely sized fragment of DNA originating from each of the three plasmids, the relative levels of retention of each of the plasmids could be determined by analysis of the restriction digest following electrophoresis on agarose gels (see Materials and Methods). Competitive initiation reactions contained *Dde*I/*Rsa*I digested pSS104, pSS110, and pBR322. RNA synthesis from these templates produced the run-off transcripts described above.

As shown in Fig. 5, the ratio of polymerase binding to the ϕ1.1B and ϕ13 promoters remained both constant and equal over the range of ionic strengths tested. This suggests that the limiting amounts of polymerase present was distributed about equally between each of the two promoters, independent of ionic strength. This same result was not observed, however, for the initiation of transcription. From Fig. 5 it is clear that under these same competitive conditions, transcription from these promoters was not initiated to similar extents as the ionic strength was increased. Instead, transcription was preferentially initiated from the class II ϕ1.1B promoter at lower ionic strengths, followed by more frequent initiation from the class III ϕ13 promoter at NaCl concentrations greater than 25 mM. These results, together with those shown in Fig. 4, would indicate that the extent of promoter binding alone is insufficient to determine the class and salt-dependent transcriptional activities of these promoters.

Initiation of Transcription from the ϕ1.1A, ϕ1.1B, and ϕ1.3 Promoters

Earlier studies have shown that transcription from class II promoters shuts down sooner and more completely than transcription from class III promoters during the infection cycle of bacteriophage T7 (10). This result has also been observed for class

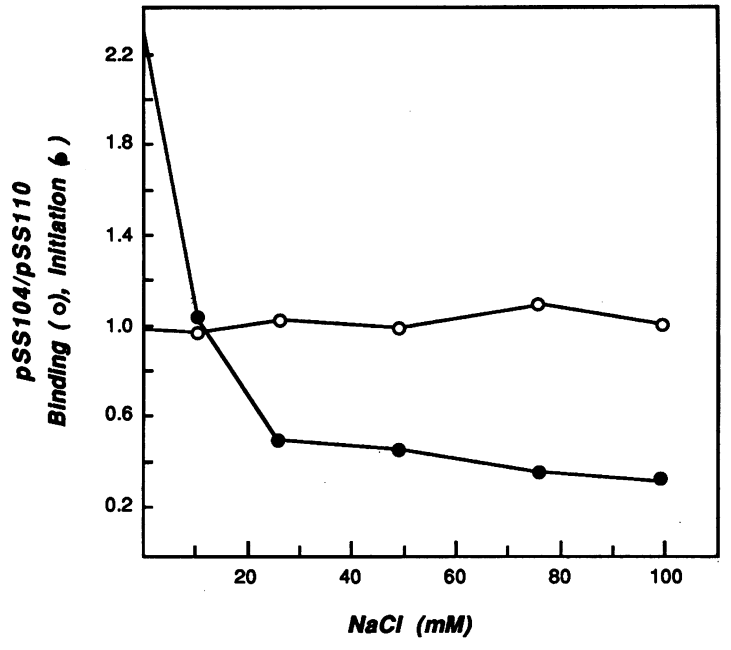

Fig. 5. pSS104/pSS110 ratios of binding and initiation of RNA synthesis by the T7 RNA polymerase. Reactions were run under competitive conditions using an excess of promoters to polymerase molecules (2.7:1) and contained the indicated amounts of NaCl. Binding reactions contained 50 units of T7 RNA polymerase and 5μg each of supercoiled pSS104, pSS110, and pBR322 DNA. Following filtration, the DNA was eluted from the filter, digested with *Rsa*I, and electrophoresed on a 1% agarose gel as described in "Materials and Methods". Binding values were corrected for nonspecific binding as described in Fig. 4. Initiation reactions contained 12.5 units of T7 RNA polymerase and 1.25μg each of *Dde*I/*Rsa*I digested pSS104, pSS110, and pBR322 DNA. The products of synthesis were electrophoresed on a denaturing (7M urea) 15% polyacrylamide gel. The levels of initiation from each promoter, as well as the levels of each plasmid retained in the binding reaction, was determined as described in "Materials and Methods". For pSS110, the 100% values for the pmoles of GTP incorporated and the μg of plasmid DNA retained are 0.034 and 0.23, respectively.

II and III promoters contained on plasmids within the infected cell (11). One exception to this, however, occurs with a plasmid which, like pAR111, contains the ϕ1.1A, ϕ1.1B, and ϕ1.3 promoters, as well as the primary origin of T7 DNA replication. In this case, transcription remained unusually strong throughout infection and thus more closely resembled that observed for class III promoters (11). To access the relative contributions to transcription of each of the promoters on pAR111, we have carried out RNA synthesis on a *Rsa*I digest of this plasmid followed by polyacrylamide gel

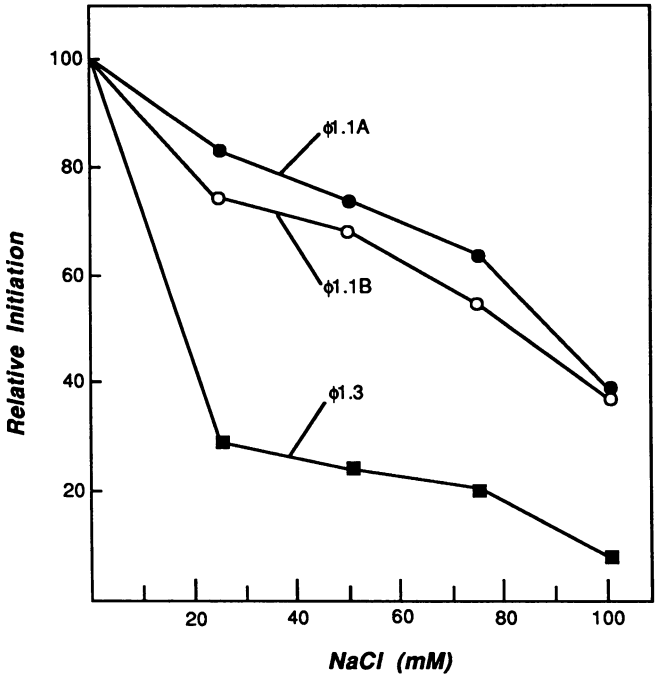

Fig. 6. Relative levels of initiation from the φ1.1A, φ1.1B, and φ1.3 promoters. The initiation of RNA synthesis was carried out by 50 units of T7 RNA polymerase in the presence of the indicated amounts of NaCl as described in "Materials and Methods" using 1 nmol of a *Rsa*I digest of pAR111 and a [^{32}P] GTP label. The products of synthesis were electrophoresed on a denaturing (7M urea) 5% polyacrylamide gel. Following autoradiography, the relative levels of initiation were determined as described in "Materials and Methods". Values of 100% for initiation (pmol GTP) from the φ1.1A, φ1.1B, and φ1.3 promoters are 0.13, 0.15, and .018, respectively.

electrophoresis of the products. This digestion places the φ1.1A and φ1.1B promoters on a fragment 653 base pairs in length and the φ1.3 promoter on a fragment 230 base pairs long. Synthesis on these fragments in the presence of [γ-^{32}P]GTP resulted in end-labeled run-off transcripts having lengths of 498, 423, and 168 nucleotides long, respectively.

Fig. 6 shows the effect of ionic strength on the relative levels of initiation from each of these promoters. Of the three promoters, φ1.3 displayed the greatest sensitivity to increased NaCl concentrations. For example, at 25mM NaCl, utilization of this promoter was decreased by over 70% while initiation from both the φ1.1A and φ1.1B promoters decreased by only about 20%. Others (22) have noted that the φ1.3 promoter is much weaker than other class II and III promoters. Since earlier studies

have suggested an important role for ionic strength in regulating transcription by the T7 RNA polymerase (see below), these results would suggest that continued transcription from this plasmid late in the T7 infection is primarily the result of RNA synthesis initiated from the ϕ1.1A and ϕ1.1B promoters. This is especially interesting since these two promoters have been implicated in the molecular mechanism for initiating the first round of DNA replication in T7 (7).

DISCUSSION

The relative simplicity of the T7 RNA polymerase, in combination with its high degree of specificity, makes it an attractive and extremely useful system for studying the basic mechanisms of protein-nucleic acid interactions. In this paper we have utilized a nitrocellulose filter binding assay and T7 RNA polymerase promoters cloned into plasmid DNAs to establish some of the fundamental characteristics of these interactions.

Nonspecific Binding

Nonspecific protein-nucleic acid binding appears to play an important and essential role in the functioning of proteins which act on DNA. In the *E. coli* RNA polymerase system, for example, it has been proposed that the rate of promoter location is greatly enhanced by the formation of nonspecifically bound complexes (23). These interactions purportedly sequester the polymerase and thus greatly reduce the volume in which the enzyme must search to locate a promoter.

To study the nonspecific binding of the T7 RNA polymerase, we have used the plasmid pBR322 which was the vector used to construct the T7 promoter-containing plasmids used in this study. At low ionic strengths, the T7 RNA polymerase was able to retain significant fractions of supercoiled, relaxed, and linear pBR322 DNA (Fig. 2). This nonpromoter binding was reduced to negligible levels in the presence of NaCl concentrations in excess of 40 mM (Fig. 1). Studies with other proteins indicate that the major component of this type of nonspecific binding is ionic in nature and involves coulombic interactions between positively charged residues on the protein and the negatively charged phosphates of the DNA (23). This conclusion is supported by the fact that supercoiled DNA was bound to an equal extent as relaxed or linear DNA even though supercoiling is known to promote strand separation and thus allow increased interactions between the enzyme and bases located within the DNA helix. Finally, we did not observe increased levels of nonspecific binding to linear DNA containing either blunt ends or 5' overhangs.

Promoter-Specific Binding

Studies with a number of proteins, including the *E. coli* RNA polymerase, have shown that a major component of sequence-specific recognition involves specific

contacts between hydrogen bond donors and acceptors which are present in the protein and DNA molecules (24). The relative insensitivity of promoter-specific binding by the T7 RNA polymerase to changes in ionic strength is consistent with such a mechanism of interaction not solely dependent on ionic interactions.

A comparison of promoter binding in supercoiled vs linear DNA molecules indicates that the binding strength, or stability, is dependent on the topological state of the DNA. In addition, we observed enhanced levels of transcription using supercoiled templates (not shown). Both of these observations are consistent with a model where the T7 RNA polymerase can more easily melt the supercoiled DNA at the promoter site forming what is commonly referred to as an open complex; that is, one where the RNA polymerase is bound to the single-stranded promoter DNA sequence. We cannot, however, infer that supercoiling has any effect on promoter specificity.

While it is currently not clear which of the bases in the T7 promoter sequence are essential for establishing promoter-specific recognition and binding by the polymerase, it is of interest that we have been unable to demonstrate promoter-specific binding by the T7 RNA polymerase to two cloned bacteriophage T3 promoters even at low ionic strength (data not shown). These promoters, present on the pBR322 derived plasmids pJB20 and pJB23 (25), have only minor sequence variations from the T7 promoters. One such variation which is thought to be important in determining the promoter specificities observed between the T7 and T3 RNA polymerases (25), occurs in the -10 to -12 region of the T3 promoter. Our inability to observe promoter-specific binding to these T3 promoters would suggest that the sequences in this region of the T7 promoter may be critical in determining promoter recognition and binding by the T7 RNA polymerase. Furthermore, this result suggests that the promoter specificities afforded these two closely related RNA polymerases occurs at the level of promoter binding.

Regulation of Transcription

Earlier work has shown that an important factor in the regulation of T7 transcription may be the ability of the T7 RNA polymerase to differentially transcribe from class II and class III promoters under conditions of varying ionic strength (11, 21, 26). These changes in intracellular ionic strength may, in turn, result from the activity of the T7 lysozyme (gene 3.5) which has been shown to be capable of altering the permeability of the host cell membrane (27). In addition, others have suggested that the preferential early transcription of class II promoters may be a result of the temporally phased entry of the T7 DNA into *E. coli* (11).

In order to characterize the contribution ionic strength has on the regulation of transcription by the T7 RNA polymerase, we have measured the levels of the

initiation of RNA synthesis using plasmids containing either a single class II (pSS104) or class III (pSS110) promoter and correlated these measurements with promoter binding (Fig. 4). For both pSS104 and pSS110, the initiation frequency, determined as the level of incorporation of [γ-^{32}P] GTP, parallels the salt-dependent transcriptional levels previously reported for other class II and class III promoters, respectively (21). Since these initiation levels may be interpreted as reflecting the levels of open complex, it would appear that the concentration of this intermediate is an important determinant of transcriptional frequency. At this stage, however, we cannot be certain that the initiation frequency is directly proportional to the concentration of open complex since DNA sequences directly downstream from the initiation site have been shown to influence transcription. For example, studies on the stability of ternary complexes composed of the T7 RNA polymerase, template DNA, and nascent RNA chains, have shown that class II promoters require the synthesis of longer RNA chains then do class III promoters in order to form complexes which are stable at high ionic strengths (26). Consistent with this result is the fact that major variations in the sequences of class II and class III promoters occur in the +2 to +6 region. In addition, while both the ϕ1.1A and ϕ1.1B promoters are located directly upstream from the primary origin of T7 DNA replication and appear to play an important role in initiating this process (7), it is not known to what extent transcription (or binding) is influenced by the proximity of these promoters to this A-T rich region.

If we assume that the open complex represents a stable polymerase-promoter intermediate, we would have expected that promoter-specific salt-dependent binding should reflect the levels of this complex and parallel the curves obtained for initiation frequency. Our results indicate, however, that initiation and binding do not always exhibit the same sensitivities to changes in ionic strength. This is clearly evident from the results of competitive binding and initiation experiments which indicate that although the polymerase remains bound to similar extents to both the ϕ13 and ϕ1.1B promoters independent of ionic strength, transcription is differentially initiated from these promoters at different ionic strengths (Fig. 5). This suggests that in addition to open polymerase-promoter intermediates, our binding curves reflect a significant level of nonproductive, yet, promoter-specific complexes. Given these results, we conclude that the extent of promoter binding alone is insufficient to determine salt and class-dependent transcriptional levels. Rather, as we have noted above, these properties appear to be more closely correlated to the level of open complex formation, suggesting that the increased rate or efficiency of formation of this polymerase-promoter intermediate, and not differential promoter binding, accounts for the preferential transcription of class III promoters in the later stages of the T7 life cycle. Further analysis, such as detailed kinetic studies of the promoter-polymerase interac-

tions, will be required in order to elucidate the relationship between the extent of promoter binding by the T7 RNA polymerase and the formation of the open complex.

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REFERENCES

1. Dunn, J. J. and Studier, F. W. (1983) *J. Mol. Biol.* **166**, 477-535.
2. Studier, F. W. (1972) *Science* **176**, 367-376.
3. Hausmann, R. (1976) *Curr. Top.* **75**, 77-110.
4. Dunn, J. J. and Studier, F. W. (1981) *J. Mol. Biol.* **148**, 303-330.
5. Studier, F. W. and Rosenberg, A. H. (1981) *J. Mol. Biol.* **153**, 503-525.
6. Saito, H., Tabor, S., Tamanoi, F., and Richardson, C. C. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3917-3921.
7. Romano, L. J., Tamanoi, F., and Richardson, C. C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4107-4111.
8. Hinkle, D. C. (1980) *J. Virol.* **34**, 136-141.
9. Studier, F.W., Dunn, J.J. (1983), *Cold Spring Harbor Symp. Quant. Biol.* **47**, 999-1007.
10. McAllister, W. T. and Wu, H.-L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 804-808.
11. McAllister, W. T., Morris, C., Rosenberg, A. H., and Studier, F. W. (1981) *J. Mol. Biol.* **153**, 527-544.
12. Studier, F. W. (1969) *Virology* **39**, 562-574.
13. Richardson, C. C. (1966) *J. Mol. Biol.* **15**, 49-61.
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory manual*, Cold Spring Harbor Press, New York.
15. Bolivar, F. and Backman, K. (1979) In *Methods in Enzymology* **68**, ed Wu, R., pp.245-276, Academic Press, New York, New York.
16. Wallace, R.B., Johnson, M.J., Suggs, S.V., Miyoshi, K., Bhatt, R., Itakura, K. (1981) *Gene* **16**, 21-26.
17. Fischer, H. and Hinkle, D. C. (1980) *J. Biol. Chem.* **255**, 7956-7964.
18. Hinkle, D. C. and Chamberlin, M. J. (1972) *J. Mol. Biol.* **70**, 157-185.
19. Strauss, H.S., Boston, R.S., Record, M.T., Burgess, R.R. (1981) *Gene* **13**, 75-87.
20. Chamberlin, M. J. and Ring, J. (1973)*J. Biol. Chem.* **248**, 2245-2250.
21. Jolliffe, L. K., Carter, A. D., and McAllister, W. T. (1982) *Nature* **299**, 653-656.
22. Panayotatos, N. and Wells, R. D. (1979) *Nature* **280**, 35-39.
23. vonHippel, P. H., Bear, D. G., Morgan, W. D., and McSwigger, J. A. (1984) *Ann. Rev. Biochem.* **53**, 389-446.
24. vonHipple, P. H., Bear, D. G., Winter, and R. B., Berg, O. G. (1982) In *Proteins: Structure and Function*, ed Rodriguez, R. L. and Chamberlin, M. J., pp. 3-33,Praeger, New York, New York.
25. Bailey, J. N., Klement, J. F., McAllister, W. T. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2814-2818.
26. McAllister, W. T. and Carter, A,. D. (1980) *Nuc. Acids Res.* **8**, 4821-4837.
27. Condit, R. C. (1975) *J. Mol. Biol.* **98**, 45-56.