An *Escherichia coli* RNA Polymerase Tight-Binding Site on T7 DNA Is a Weak Promoter Subject to Substrate Inhibition†

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ABSTRACT: A specific *Escherichia coli* RNA polymerase tight-binding (TB) site on bacteriophage T7 has been located at 32988 base pairs from the left end of T7. This site is referred to as the T7 F promoter since it is fully active in vitro. Kinetics of association and dissociation have been measured by use of the abortive initiation assay and runoff transcription. The association constant, $k_a \approx 9 \times 10^5$ M^{-1} s^{-1}, is of the same magnitude as k_a for the T7 minor promoters. In competitive titration assays, the F promoter was found to be slightly weaker than the minor T7 E promoter at low RNA polymerase concentrations and, as expected, much weaker than the major T7 A3 promoter. An unusual RNA polymerase mediated inhibition of both the association rate and the transcriptional activity was observed at moderately high concentrations of polymerase. A mechanistic model analogous to enzyme substrate inhibition is presented.

The *Escherichia coli* RNA polymerase (RNAP)[1] binds to DNA in vitro in a variety of modes ranging from totally nonsequence-specific electrostatic binding to highly specific binding at promoter sites. Promoter site binding is relatively well characterized [see Rodriquez & Chamberlin (1982) and McClure (1985)] and is an extremely strong interaction ($K \approx 10^{11}$ M^{-1}). Nonspecific binding has been shown to occur at DNA ends with a binding affinity ($K \approx 10^8$ M^{-1}) about 600-fold stronger than that at random internal sites (Melancon et al., 1983). Stable site-specific binding to nonpromoter sequences has also been observed. These sites are collectively referred to as tight-binding (TB) sites (Kadesch et al., 1980; Melancon et al., 1982). Some bacteriophage T7 restriction fragments have been reported to carry transcriptionally active TB site (Melancon et al., 1982). Presumably, these sites have sequences that to some extent resemble promoters, but no individual TB site has ever been specifically identified or characterized. It has not been established whether TB sites play a significant function in vivo, in terms of reducing the concentration of free polymerase, hindering one-dimensional diffusion of proteins along the DNA or possibly enhancing binding of polymerase to nearby promoter sites. At the very least, quantitative in vitro thermodynamic and kinetic analyses of RNAP–promoter interactions require a thorough knowledge of competing nonpromoter interactions.

Measurement of RNAP affinities for DNA fragments has been performed by two classes of methods. Filter binding techniques and related methods are independent of transcription and thus measure binding directly, but the data obtained are often difficult to interpret unambiguously. Other methods are dependent upon transcription and normally involve measurement of initiation of transcription as an indirect assay of binding. Binding can be reduced conceptually to two minimum steps: an initial binding of RNAP to DNA to form the initial complex RP_i followed by isomerization of RP_i into the open complex RP_o. In the presence of nucleotides, RP_o is capable of initiation. This is written schematically as shown in Scheme I, where R represents RNAP, P is the promoter, k_2 is the isomerization rate for conversion of initial complex (RP_i) to open complex (RP_o), and $K_B = k_1/k_{-1}$ is the equilibrium binding constant for the formation of RP_i. Well-established techniques employ measurement of product in the form of abortive initiation turnover (McClure, 1980) or runoff transcription (Stefano & Gralla, 1980) as a function of time to determine K_B, k_2, and k_{-2} independently. So far, only sites known to support substantial transcription in vitro have been kinetically characterized this way. Scheme I is most certainly an oversimplification. It is clear from in vitro temperature dependence studies that for the λ P_R (Roe et al., 1984, 1985) and lac UV5 (Buc & McClure, 1985) promoters the RP_i to RP_o isomerization consists of at least two steps, the second in rapid equilibrium at 37 °C. The observed terms K_B and k_2 are thus composites of elementary rate constants. Nevertheless, all evidence to date supports the general assumption that in vivo promoter strength is proportional to $k_a = K_B k_2$, independent of the mechanistic interpretation of these terms.

Scheme I

$$R + P \underset{k_{-1}}{\overset{(K_B)\ k_1}{\rightleftharpoons}} RP_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o \xrightarrow[NTP]{k_3} \text{product}$$

Using computer-assisted sequence analysis, we have searched the entire T7 sequence for promoter-like sequences. A site at base pair 32988 closely resembled the consensus sequence for promoters (Figure 1). We have isolated this site on two fragments, a 716-bp *Hae*III fragment (32712–33428 bp) and a 498-bp *Ava*II subfragment of the 716-bp fragment. We call this site the F promoter since it does promote transcription in vitro nearly as well as the T7 minor promoters B, C, D, and E. We identified the start point of transcription, measured the kinetic parameters for binding by RNAP, and carried out competitive in vitro titrations of the TB site (F) and a minor promoter (E). Competitive titrations of the E promoter and the major A3 promoter are also presented for comparison.

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[1] Abbreviations: RNAP, RNA polymerase; DTT, dithiothreitol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); nt, nucleotide(s).

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	-35 TTGACA	-10 TATAAT	+1	
AAAAAGAGTA	TTGACT	TAAAGTCTAACCTATAG	GATACT	TACAGCC ATCGAGAG A1
AAAACAGGTA	TTGACA	ACATGAAGTAACATGCAG	TAAGAT	ACAAATC GCTAGGTA A2
AACAAAACGG	TTGACA	ACATGAAGTAAACACGG	TACGAT	GTACCAC ATGAAACG A3
GATAAGCAAC	TTGACG	CAATGTTAATGGGCTGA	TAGTCT	TATCTT ACAGGTCA C
AAGATAGGCG	TTGACT	TGATGGGTCTTTAGGTG	TAGGCT	TTAGGT GTTGGCTT D
CTTACGGATG	ATGATA	TTTACACATTACAGTGA	TATACT	CAAGGCC ACTACAGA E
GATGTCACCA	TTGACA	CGGCGGTCATACGCTGG	CATGAT	GCGGAAC ATATCGAA F
AGGTCATTCA	CTGAGA	ATTGCTGTCCGTCTGG	CATAGT	GATGGAT AGGTCCGA *
CCATCGAATG	GCGCAA	AACCTTTCGCGGTATGG	CATGAT	AGCGCCC GGAAGAGA Lac i

FIGURE 1: Promoter sequences. T7 promoter sequences are taken from Siebenlist (1979) (A1, A2, and A3), McConnell (1979) (C), Dunn and Studier (1981) (D), and Dunn and Studier (1983) and Prosen and Cech (1985) (E). lac i promoter sequence is taken from Calos (1978). The sequence denoted ((*)) corresponds to a proposed inhibitory binding site at 32 928 bp. The most frequently observed bases in the two consensus regions (Rosenberg & Court, 1979; Siebenlist et al., 1980) are shown for comparison. Position +1 corresponds to the transcription initiation site. Promoter sites are identified in the text by the location of the rightmost conserved T in the underlined −10 consensus hexamer.

Materials and Methods

Polymerase and DNA. RNA polymerase (RNAP) was isolated from *E. coli* (Grain Processing) by the methods of Burgess and Jendrisak (1975). Polymerase activity was measured periodically by the T7 template functional assay of Chamberlin et al. (1979). Activity ranged from 40 to 80% of total concentration. Total polymerase concentration is reported here, unless otherwise indicated. Bacteriophage T7 was grown in *E. coli* and the DNA isolated by standard procedures (Cech & McClure, 1980). Poly[d(IC)·d(IC)], heparin, and CpA were purchased. Nucleotides for transcription were purchased (P-L Biochemicals or Sigma) and purified as described (McClure et al., 1978). Fragments of DNA created by restriction endonuclease digestion were isolated after gel electrophoresis by electroelution of excised DNA fragment bands. Contaminating acrylamide was removed by loading DNA solutions onto DEAE-A25 Sephadex columns and rinsing with 0.1 M KCl. DNA was dialyzed into 25 mM KCl, 10 mM Tris, pH 8 at 25 °C, and 1 mM EDTA. Promoter-containing fragments were identified by use of Delila computer programs (Schneider et al., 1982) to search T7 sequence data (Dunn & Studier, 1983) for restriction nuclease cleavage sites. The identity of the fragments was verified by restriction pattern analysis of isolated fragments. Fragments referred to this paper are shown in Figure 2. Two nomenclatures are used to designate positions on the T7 genome: base pairs from the left end (1–39 936) and T7 units (0–100, where 0 is the left end and 100 is the right end of the T7 genetic map) (Dunn & Studier, 1983). Promoter sites are specified by the base pair corresponding to the highly conserved T at the downstream end of the −10 region consensus hexamer.

Abortive Initiation Assays. Abortive initiation assay procedures have been described earlier (Johnston & McClure, 1976; Cech et al., 1980). The primary abortive initiation products used in these kinetic studies were CpApU, pApC, and CpApU for the F, E, and A3 promoters, respectively. Other abortive initiation products used are as stated in the text. The transcriptional buffer used for abortive initiation and transcription reactions was buffer KCl-80 (80 mM KCl, 40 mM Tris, pH 8, 10 mM $MgCl_2$, and 1 mM DTT). For salt-dependence studies, the buffers were as above except the concentration of KCl was varied from 0 to 201 mM.

To measure association constants, the dinucleoside monophosphate CpA (1 mM) and the elongating nucleoside triphosphate UTP (0.04 mM), with [α-^{32}P]UTP added at 2000 cpm/pmol of UTP, were preincubated for 10 min at 37 °C with 1 nM DNA template. Polymerase, ranging in concentration from 25 to 300 nM, was then added at time zero, and aliquots of the reaction were measured for product formation as a function of time. Control reactions were performed by preincubating RNAP with the DNA template for 10 min to allow RP_o formation. Nucleotides were then added at time zero and aliquots of the reaction measured for product formation as a function of time.

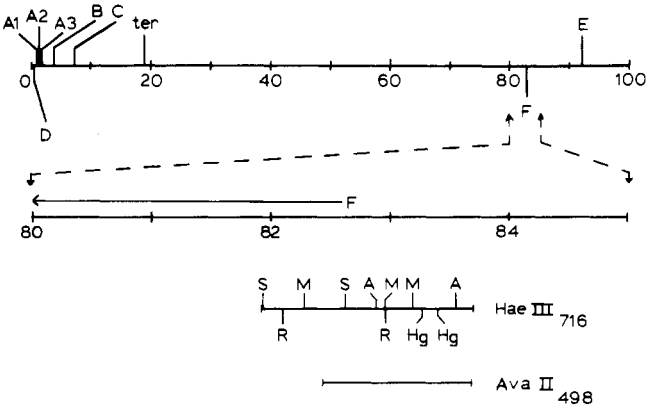


FIGURE 2: Transcription map of T7 for *E. coli* RNA polymerase. The A promoters are active in vivo early in infection. B, C, D, and E promoters are only known to transcribe in vitro. The genome is normalized to 100 units (1 T7 unit = 399.36 bp). Restriction fragments used in this work are also shown. Fragment lengths in base pairs (shown as subscripts) were determined from computer-assisted analysis of DNA sequence (Dunn & Studier, 1983). Subsequent verification of fragment identity was determined by restriction patterns of isolated fragments. Some restriction endonuclease cleavage sites on the $HaeIII_{716}$ and Ava_{II498} fragments are *Alu*I (A), *Dde*I (D), *Fnu*4HI (F), *Hae*III (H), *Hgi*AI (Hg), *Mbo*II (M), *Rsa*I (R), and *Sfa*NI (S).

To obtain dissociation rates, the methods described by Cech and McClure (1980) were followed. The value of k_{-2} was measured by preincubating 160 nM RNAP with 1 nM template (final concentrations) for 10 min at 37 °C prior to addition of heparin (final concentration 100 μg/mL) or poly[d(IC)·d(IC)] [0.04 mM phosphate; see Dayton et al. (1984)] as the sequestering agent, at time zero. Aliquots were removed at later times and mixed with nucleotides in reaction buffer KCl-80 for 5 min. The amount of product from the 5-min reactions is proportional to the amount of remaining open complex, RP_o.

Runoff Transcription. Runoff transcription reactions were performed in buffer KCl-80, unless otherwise noted. The final nucleotide reaction concentrations were 400 μM each of ATP, GTP, and CTP and 10 μM UTP with [α-^{32}P]UTP at 20 000 cpm/pmol of UTP. RNAP was preincubated for 10 min with promoter-containing DNA fragment at the final concentrations designated in figures. Nucleotides were then added to initiate the reaction. After 0.5 min, heparin was added to 40 μg/mL to prevent further reinitiation. The reaction was allowed to proceed for an additional 10–15 min to assure that all transcription was complete. All reactions were stopped by addition of EDTA to 20 mM. Urea was added immediately to 7 M, and the reaction tube was heated to 90 °C for 3 min to denature RNA. Transcripts were analyzed on 7 M urea–5% polyacrylamide gels.

Titrations. Methods of Cech and McClure (1980) were used to determine saturating concentrations of RNAP for each DNA template fragment. RNAP at various concentrations was preincubated with 1 nM DNA template for 10 min. Transcription was initiated by addition of nucleotides, and

turnover rates were determined 7.5 and 15 min or 10 and 20 min after initiation. Runoff transcription reactions were similar to those described above in the previous section except that the RNAP concentration was varied as designated. Relative amounts of runoff transcripts were subsequently determined by scintillation counting of radioactive RNA in each excised gel band. Competitive titrations were performed by preincubating two different promoter-containing fragments, each at 1 nM final concentration, with varying concentrations of RNAP for 10 min. Reactions were initiated with the addition of nucleotides. Promoter activities could be individually quantitated in the presence of the other promoter by virtue of the fact that each had a unique abortive initiation product or length of runoff transcript.

Analysis of Data. Data were fitted to nonlinear equations as described by Prosen and Cech (1985) and equations described in the text. Nonlinear least-squares programs utilized the Marquardt algorithm (Bevington, 1969). Since confidence intervals are often asymmetric for nonlinear equatons (Johnson, 1983), a statistical F test was used to determine the values of the parameters at a 67% confidence level (Bevington, 1969). The kinetic parameters k_a and K_B were calculated with active RNAP concentrations.

RESULTS

Location. Two computer sequence analysis methods were used as previously described (Prosen & Cech, 1985) to search the entire T7 genome for potential RNAP binding sites. More than 600 sites on T7 were identified with a direct method that simply searched for the consensus sequence

TTGACA...16–18 bp...TATAA*T*

allowing up to four mismatches. About half these sites contained the highly conserved italicized T. However, only eight sites were found that had less than three mismatches. Only two of these sites, the A3 and the 32 988 site, had the optimal spacing of 17 bp as well. For comparison, the other sequenced T7 promoters (Figure 1) have three or four mismatches with 17-bp spacing, except A2 which has two mismatches and 18-bp spacing. A second method, which incorporated the pattern-learning algorithm to separate functional from nonfunctional sequences (Stormo et al., 1982), located approximately 100 sites on T7. Unfortunately, this approach generated "learned" patterns that ranked sites quite differently depending on the number of base pairs included in the pattern and whether the source of nonfunctional sequences was bacteriophage or *E. coli* in origin. In general, the pattern-learning method appeared to sacrifice the ability to accurately rank sites in order to completely separate functional from presumably nonfunctional sites (D. Kemme and C. Cech, unpublished results). The 32 988 site was ranked high by most patterns, however, typically in the top 10.

The predicted direction of transcription from the RNAP binding site at 32 988 is toward the left end of T7 (Figure 2). The site was isolated on two different restriction fragments, the *Hae*III$_{716}$ fragment (32 712–33 428 bp) and the *Ava*II$_{498}$ fragment (32 915–33 413 bp, contained within *Hae*III$_{716}$). These two fragments were analyzed for ability to support runoff transcription. The major runoff transcripts from the *Hae*III$_{716}$ and the *Ava*II$_{498}$ fragments were 272 and 62 nucleotides (nt), respectively (Figure 3). These runoff transcripts are consistent with a promoter near 32 988 transcribing toward the left end of T7.

The *Hae*III$_{716}$ fragment was digested with various restriction enzymes (Figure 2). DNA from each digest listed in Figure 3 was transcribed in buffer KCl-80. The major runoff transcript in each lane was consistent with transcription initiating at 32 980 ± 10 bp. Runoff transcript lengths were approximately 272 nt from the undigested *Hae*III$_{716}$ fragment and the *Alu*I digest-transcription reactions, 275 nt from the *Hgi*AI digest-transcription reaction, 210 nt from the *Rsa*I digest-transcription reaction, and 118 nt from the *Mbo*II digest-transcription reaction. Transcription of the *Ava*II$_{498}$ fragment yielded a major transcript of 62 nt and a minor transcript of 250 nt (4:1 molar ratio; lane 9 of Figure 3). The 250-nt runoff transcript most likely initiates near the center of the fragment or initiates at an end and terminates near the center. These digests and a *Sfa*NI digest of the *Hae*III$_{716}$ fragment were compared with respect to abortive production of CpApU (see below). *Sfa*NI cleaves the DNA at 32 716 and at 32 986 bp, in the presumed binding site. The abortive initiation product CpApU was produced from both isolated fragments (*Hae*III$_{716}$ and *Ava*II$_{498}$) and from all digest-abortive initiation reactions except the *Sfa*NI digest-abortive initation reaction. Since the *Ava*II$_{498}$ fragment does support CpApU production but does not contain the 32 716-bp *Sfa*NI site, restriction at 32 986 must be the cause of promoter inactivation.

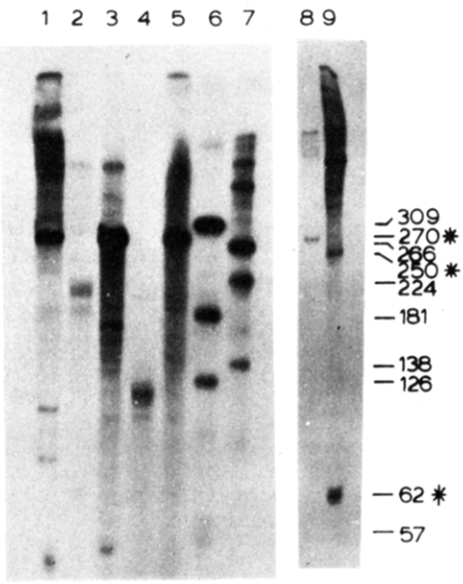


FIGURE 3: Runoff transcription from *Hae*III$_{716}$ fragment and fragments derived from *Hae*III$_{716}$. The following restriction fragments were used as templates: (1) *Hae*III$_{716}$ digested with *Hgi*AI; (2) *Hae*III$_{716}$ digested with *Rsa*I; (3) *Hae*III$_{716}$ digested with *Alu*I; (4) *Hae*III$_{716}$ digested with *Mbo*II; (5) *Hae*III$_{716}$; (6) 711-bp *Dde*I fragment; (7) 763-bp *Hin*fI fragment; (8) *Hae*III$_{716}$; (9) *Ava*II$_{498}$. Fragments in lanes 6 and 7 each contained the A1, A2, A3, and D promoters. Transcripts from these fragments were used as RNA standards. Standards are, from lane 6, 309-nt A1 transcript, 181-nt A2 transcript, 126-nt D transcript, 57-nt A3 transcript and, from lane 7, 266-nt A1 transcript, 224-nt D transcript, and 138-nt A2 transcript. Transcript lengths designated • were calculated relative to the standards. All reactions were run in buffer KCl-80. DNA concentrations were 4 nM DNA template in lanes 1–5 and 9, 2 nM in lane 6, and 1 nM in lanes 7 and 8. RNA polymerase concentrations were 120 nM active RNA polymerase in lanes 1–7, 36 nM in lane 8, and 20 nM in lane 9.

The predicted 5′-end of the F promoter transcript is pppApU, which was verified by use of the abortive initiation assay (Johnston & McClure, 1976; Cech et al., 1980). The *Hae*III$_{716}$ fragment produced a somewhat low but significant amount of pApU product [turnover rate of 14 nM product min^{-1} (nM DNA)$^{-1}$], essentially no pApC (turnover rate of 2), and no pppApC or pppCpA (turnover rate less than 1). In a reaction using CpA and UTP, however, the fragment supported CpApU production with a turnover rate of 155. An

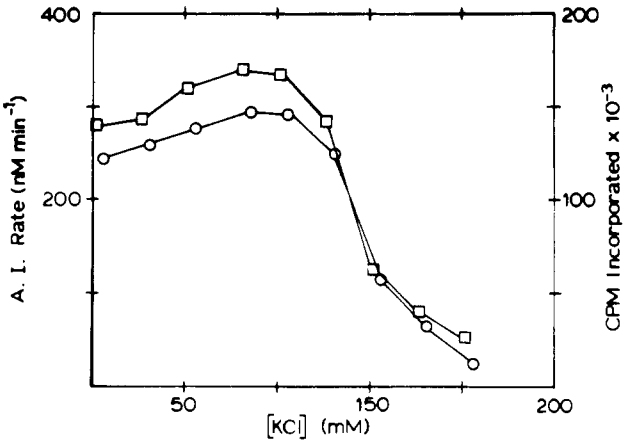


FIGURE 4: Salt dependence of T7 F promoter activity. Buffers for the F promoter abortive initiation (□) and runoff transcription (○) were the same as buffer KCl-80 except the KCl concentration varied from 1 to 206 mM. Abortive-initiation reactions were performed by preincubating 1 nM *Hae*III$_{716}$ with 80 nM RNA polymerase for 10 min and then initiating the reaction with the addition of CpA and UTP as described under Materials and Methods. Reactions were analyzed 5.5 and 10 min after initiation. Runoff transcription reactions were performed as described under Materials and Methods under identical conditions.

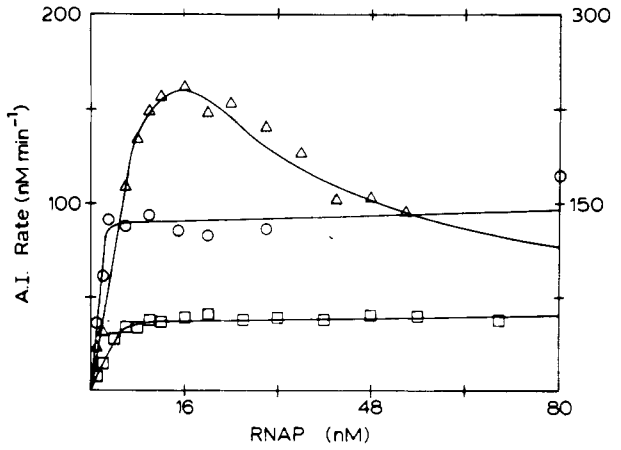


FIGURE 5: Abortive-initiation titrations of the A3, E, and F promoters by RNA polymerase. For each titration, one template at 1 nM was preincubated 10 min with RNA polymerase at concentrations shown. The *Hae*III$_{716}$ F promoter activity (△) is given by the right-hand scale; the A3 *Hpa–Hae*$_{810}$ (○) and the E *Hin*fI$_{671}$ (□) promoter activities are given by the left-hand scale. Plateau turnover rates are dependent on the promoter (Dayton et al., 1984) and choice of product (Prosen & Cech, 1985) but nevertheless reflect 100% open complex formation. All reactions were performed in buffer KCl-80 as described under Materials and Methods.

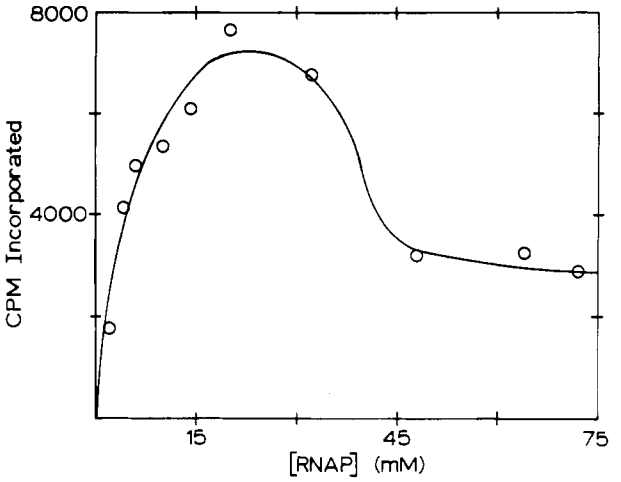


FIGURE 6: Runoff transcription titration of the *Hae*III$_{716}$ F promoter as a function of [RNAP]. The *Hae*III$_{716}$ fragment (1 nM) was preincubated 10 min with RNAP at concentrations shown. Reactions were performed as described under Materials and Methods, quenched with EDTA (20 mM) after 13 min of transcription, and analyzed on a 7 M urea–5% polyacrylamide gel. Excised gel bands were counted in a scintillation counter to determine relative amounts of transcription in each reaction.

oligonucleotide was produced with ATP and UTP that was consistent with the expected tetramer. These results along with the results of the *Sfa*NI digest-abortive initiation reaction strongly suggest the F promoter is at position 32 988 and produces an RNA beginning with pppApU at position 32 980.

Salt Dependence. Prosen and Cech (1985) showed that for the E promoter increasing ionic strength had a greater inhibitory effect on abortive-initiation turnover (maximum rate near 0 mM KCl) than on runoff transcription (maximum synthesis near 150 mM KCl). There appeared to be no such differential effects of ionic strength for the F promoter (Figure 4). Both the maximum abortive-initiation turnover rate and the maximum rate of runoff transcription occurred near 80 mM KCl.

RNAP Inhibition and Kinetics. An unusual inhibition of transcription caused by RNAP itself became apparent during kinetic studies of the F promoter. It was observed that increasing [RNAP] significantly decreased the amounts of transcription at concentrations that normally produce a saturating plateau of transcriptional activity (McClure et al., 1978; Cech & McClure, 1980). The observation was verified with the use of two separate RNAP preparations, two fragment preparations (*Hae*III$_{716}$ and *Ava*II$_{498}$), and two transcriptional assays (abortive initiation and runoff transcription).

RNAP titration experiments for the F promoter were performed by the methods of Cech and McClure (1980). Abortive-initiation turnover as a function of RNAP concentration for the *Hae*III$_{716}$ fragment is shown in Figure 5. Similar titrations of the A3 and E promoters with the same RNAP solutions (Figure 5) showed saturation at low RNAP concentrations followed by a plateau of activity. In contrast, the F promoter activity rose more slowly as a function of RNAP concentration and never reached a plateau. A maximum in activity was observed followed by a decrease at higher RNAP concentrations. The turnover rate at 54 nM was only 55% the turnover rate at 10 nM. Turnover from the *Ava*II fragment at 54 nM RNAP was 60% the turnover rate for a reaction at 10 nM RNAP (data not shown). Transcriptional efficiency for runoff transcription from the *Hae*III$_{716}$ fragment at 48 nM RNAP was only 40% the efficiency for a reaction with 20 nM RNAP (Figure 6).

The rate of association of RNAP to the *Hae*III$_{716}$ fragment also decreased when RNAP was increased above 40 nM (Figure 7a). This inhibition of the association rate did not appear to occur for experiments performed with the *Ava*II$_{498}$ fragment (Figure 7b). Although data for *Ava*II$_{498}$ were well represented by a rectangular hyperbola (i.e., the association rate appeared not to be inhibited by increased RNAP), the turnover rates for each lag time experiment decreased as RNAP increased (transcriptional inhibition). If it is assumed that the rate of association was unaffected by transcriptional inhibition, kinetic parameters derived from the data for the 498-bp fragment in Figure 7b were $K_B = 2.2 \times 10^7$ M^{-1}, $k_2 = 0.030$ s^{-1}, and $k_a = 6.6 \times 10^5$ M^{-1} s^{-1}. Calculation of the 67% confidence levels for k_a yielded upper and lower limits of 9.2×10^5 and 4.9×10^5 M^{-1} s^{-1}, respectively. However, this analysis provides only rough estimates of kinetic parameters. A model for inhibition is presented below along with estimates of the inhibition constants and the kinetic parameters

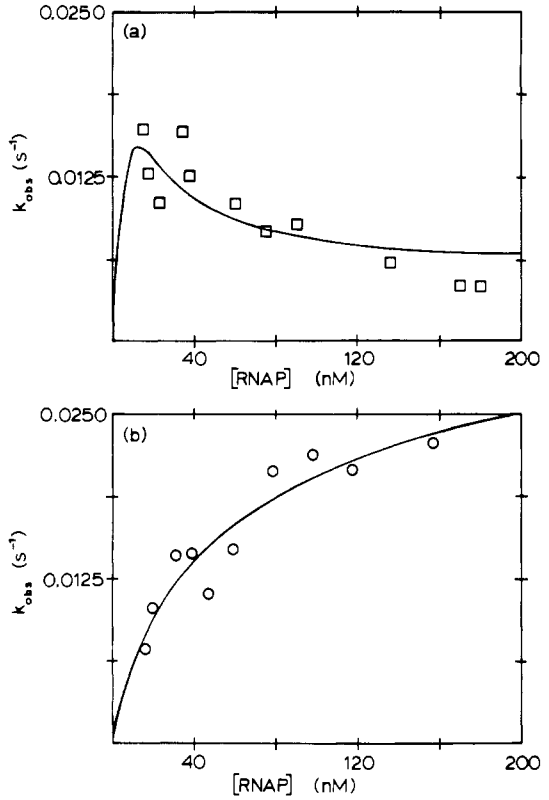


FIGURE 7: Nonlinear k_{obsd} plots for (a) *Hae*III$_{716}$ and (b) *Ava*II$_{498}$ fragments. Curves were fit to eq 1 by nonlinear regression analysis with K_{I2} values set at 2.1×10^7 M^{-1} for *Hae*III$_{716}$ and 1.6×10^7 M^{-1} for *Ava*II$_{498}$. Active RNA polymerase concentrations are indicated.

in the presence of concurrent inhibitory RNAP binding.

An experiment was performed with 1 nM *Ava*II$_{498}$ fragment to determine if inhibition was reversible. This was accomplished by diluting [RNAP] from 52 to 26 nM after initiation of the reaction (Figure 8a). The turnover rate increased to the rate obtained in a 26 nM RNAP control reaction with an estimated lag of $t_{1/2} \approx 3$ min. A complementary experiment, in which polymerase concentration was increased from 26 to 52 nM (Figure 8b) after initiation, was performed to determine whether the reduction of transcriptional inhibition observed in titration experiments could be explained simply by the reduction in the association rate at high polymerase concentrations. After preincubating template with polymerase at 26 nM for 10 min, CpA and UTP were added at time zero, and steady-state product turnover was monitored for 6 min. A small aliquot of polymerase was then added, bringing total [RNAP] to 52 nM without significantly diluting the reaction. A new steady-state turnover rate, identical with that of a control reaction initiated after 10-min preincubation at 52 nM RNAP, was quickly obtained. The apparent half-time for establishment of the reduced turnover rate was 21 s (k_{obsd} = 0.033 s^{-1}). Inhibition of transcriptional activity in titration experiments therefore appears to be a phenomenon distinct from reduction of association rate.

Concurrent binding of a second RNAP molecule to RP_o, presumably at an adjacent noncompeting site on the DNA, is sufficient to explain the observed transcriptional inhibition. To describe the inhibition of the rate of association, inhibitory binding of a second RNAP molecule to RP_c is sufficient. Thus, Scheme II describes both types of observed inhibition. In Scheme II, RRP_i and RRP_o are the inhibition complexes formed from the closed and open complexes, respectively; K_{I1} and K_{I2} are the inhibition constants for the formation of the inhibitory complexes. This scheme is analogous to substrate

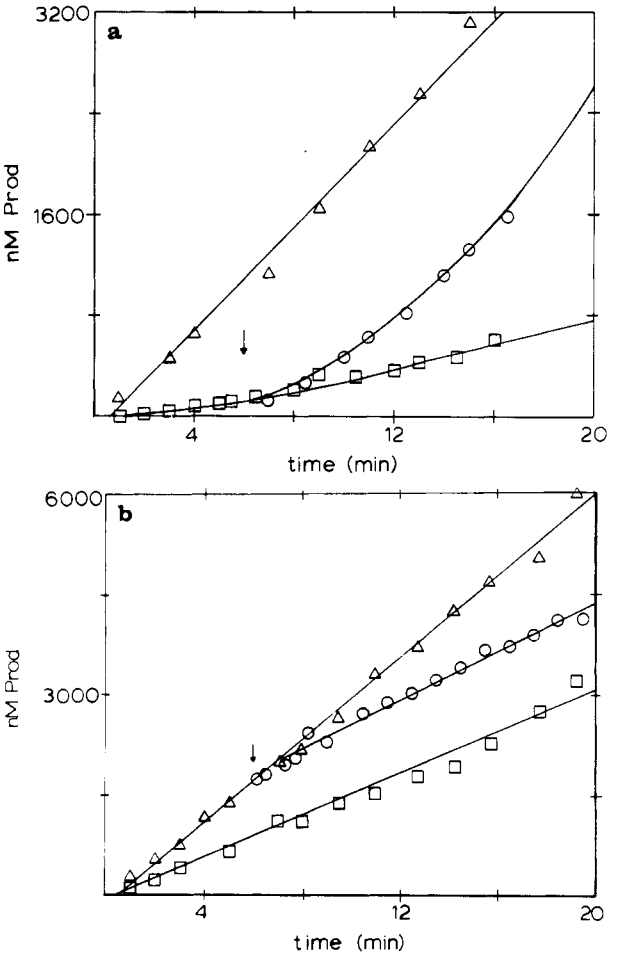
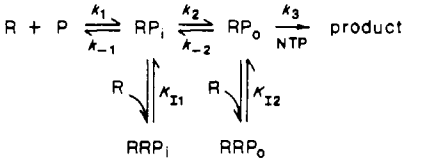


FIGURE 8: (a) Reverse of RNA polymerase mediated inhibition. The control reaction (△) contained 1 nM *Hae*III$_{716}$ and 26 nM RNAP, which were preincubated for 10 min before the addition of nucleotides to initiate the reaction. The inhibited reaction (□) was the same as the control except [RNAP] was 52 nM. At the time indicated by the arrow, an aliquot of the inhibited reaction was diluted into a solution containing buffered DNA (○). The only concentration that changed during the dilution was that of RNA polymerase, from 52 to 26 nM. (b) RNA polymerase concentration jump and resulting increase in transcription inhibition. Control reactions containing 1 nM *Hae*III$_{716}$ and 26 (△) or 52 nM RNAP (□) were preincubated 10 min before initiation by addition of nucleotides. At the time indicated by the arrow, additional RNAP was added to an aliquot of the 26 nM reaction, increasing [RNAP] from 26 to 52 nM without significantly altering the concentration of other reaction components (○).

inhibition in enzyme kinetics, where a second substrate molecule binds two intermediates. Since RNA polymerase is in excess, it is considered the substrate in this case.

Scheme II



The following equations can be derived from Scheme II, when [R] ≫ [P] and $k_2 \gg k_{-2}$, when the inhibition complexes are in equilibrium with the closed and open complexes, and when steady state is assumed for RP_i ($d[RP_i]/dt = 0$):

$$k_{obsd} = \frac{k_2 K_B[R](1 + K_{I2}[R])}{1 + k_2 + K_B[R](1 + K_{I1}[R])} \quad (1)$$

$$[\text{product}] = \frac{k_3 t}{1 + K_{I2}[R]} \quad (2)$$

Table I: Kinetic Parameters of F Promoter on $AvaII_{498}$ and $HaeIII_{716}$ Fragments

parameter	$AvaII_{498}$[a]	$HaeIII_{716}$[a]
k_2 (s^{-1})	0.014	0.22
K_B (M^{-1})	6.2×10^7	1.4×10^7
k_a (M^{-1} s^{-1})	8.6×10^5	3.0×10^6
K_{I1} (M^{-1})	6.4×10^6	8.4×10^8
K_{I2} (M^{-1})	1.6×10^7	2.1×10^7
k_{-2} (s^{-1})	1.4×10^{-3}	1.4×10^{-3}
$K_{eq} = k_a/k_{-2}$ (M^{-1})	6×10^8	2×10^9

[a] Kinetic parameters were determined by use of nonlinear regression analysis of k_{obsd} vs. [RNA polymerase] and [product] vs. [RNA polymerase] data according to eq 1 and 2, respectively, or k_{off} data for k_{-2}. Errors are discussed in the text.

Equation 2 describes [product] as a function of time and as a function of excess [R] for a titration experiment, where RNAP has been preincubated with the promoter for 10 min. Since [R] must be in excess, titration data in Figure 5 (1 nM DNA) were fit to eq 2 with only data for [R] > 10 nM. The values obtained by a nonlinear fit were for $HaeIII_{716}$ $K_{I2} \approx 2.1 \times 10^7$ M^{-1} and for $AvaII_{498}$ $K_{I2} \approx 1.6 \times 10^7$ M^{-1}. By inserting these K_{I2} values into eq 1, the association data from Figure 7 could be fitted to yield the following: for $HaeIII_{716}$, $K_B = 1.4 \times 10^7$ M^{-1}, $k_2 = 0.22$ s^{-1}, $k_a = 3.0 \times 10^6$ M^{-1} s^{-1}, and $K_{I1} = 8.4 \times 10^8$ M^{-1}; for $AvaII_{498}$, $K_B = 6.2 \times 10^7$ M^{-1}, $k_2 = 0.014$ s^{-1}, $k_a = 8.6 \times 10^5$ M^{-1} s^{-1} (within 67% confidence interval obtained assuming no inhibition, see above), and $K_{I1} = 6.4 \times 10^6$ M^{-1} (Table I). The k_2 and K_B values but not k_a values were sensitive to the values of the inhibition constants.

Experiments to determine the dissociaiton rates were performed with both the $HaeIII_{716}$ and $AvaII_{498}$ fragments. No difference in the rates of dissociation was observed for the two fragments. Assuming $k_{-1} \gg k_2$ and ignoring RNAP-mediated inhibition (see Discussion), k_{-2} equals the dissociation rate, k_{off}, observed when RP_o is challenged with a sequestering agent for free polymerase (McClure, 1980; see Materials and Methods). The half-life of dissociation was determined to be 3.0 min [$k_{-2} = (3.8 \pm 1.1) \times 10^{-3}$ s^{-1}] with heparin and 8.2 min [$k_{-2} = (1.4 \pm 0.3) \times 10^{-3}$ s^{-1}] with poly[d(IC)·d(IC)] as sequestering agent. The theoretical binding constant for RP_o, $K = k_a/k_{-2}$, calculated with the poly[d(IC)·d(IC)] data for k_{-2}, is then 2×10^9 M^{-1} for the 716-bp fragment and 6×10^8 M^{-1} for the 498-bp fragment.

Promoter Strength Comparisons. Direct comparison of in vitro promoter strengths was accomplished by titrating one promoter with increasing concentrations of RNAP in the presence of a second promoter. The competitive titrations were assayed by runoff transcription and/or abortive initiation.

Figure 9 shows a runoff transcription titration of 1 nM each of F and E promoters on $HaeIII_{716}$ and $HinfI_{671}$ fragments, respectively. At low [RNAP] (lanes 3–5), the runoff transcript from the E promoter (160 nt) was transcribed more efficiently than the F promoter (270 nt). The E promoter, with $K_a = 1.2 \times 10^6$ M^{-1} s^{-1} (Prosen & Cech, 1985), is therefore stronger in vitro than the F promoter (k_a estimates ranging from 6.6×10^5 to 3×10^6 M^{-1} s^{-1}, depending on inhibition assumptions and fragment used.) Similar results were obtained with the abortive-initiation assay (data not shown). We would have liked to extend these titrations to 0.1 nM RNAP or less where relative promoter occupancy is proportional to the ratio of k_a values, but we could not obtain sufficient product to quantitate activity accurately.

The strengths of the E and A3 promoters were directly compared by the abortive-initiation assay. The results (Figure 10) show the A3 promoter ($k_a > 4 \times 10^7$ M^{-1} s^{-1}, Prosen & Cech, 1985) was nearly saturated before the E promoter was

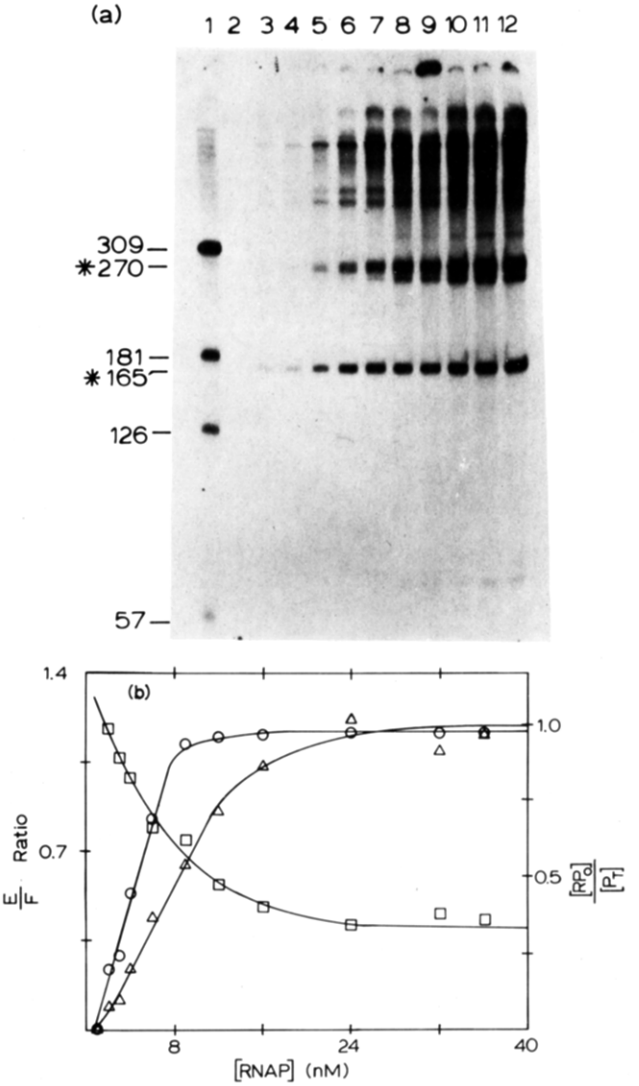


FIGURE 9: Competitive titration of E and F by runoff transcription. $HaeIII_{716}$ and $HinfI_{671}$ fragments were mixed, each at 1 nM, and preincubated 10 min with RNAP at concentrations indicated. Reactions were performed otherwise as described in Figure 6. Transcript lengths were 160 (E) and 270 nt (F). (a) Autoradiogram of runoff competitive titration. Lane 1 contained transcripts from a 711-bp *Dde*I fragment for use as standards (see Figure 3). Other lanes contained the following final concentrations of RNA polymerase: (2) 1, (3) 2, (4) 3, (5) 4, (6) 6, (7) 9, (8) 12, (9) 16, (10) 24, (11) 32, and (12) 36 nM. (b) Ratio of E transcript to F transcript total incorporated radioactivities (□) and normalized promoter occupancy, [RP_o]/[total promoter], for E (○) and F (△) promoters calculated assuming the plateau of incorporation represented 100% open complex formation.

able to sequester any RNAP. Runoff transcription comparisons could not be quantitated because of size similarities between the A3 runoff transcript and E and F fragment end-to-end transcripts. The A3 and F promoters could not be directly compared by abortive initiation because both make CpApU.

Discussion

Location. A specific site on T7 belonging to the class of sites collectively termed tight-binding (TB) sites has been located and characterized. This site is referred to as the F promoter since it is fully active transcriptionally in vitro. As determined by transcription studies, the location of the F promoter is at 32 988 bp from the left end of T7 and is consistent with earlier observations of tight-binding sites. Electron microscopy studies of T7 (Koller et al., 1978) showed higher than basal level binding in a region near 83.0 T7 units (33 000

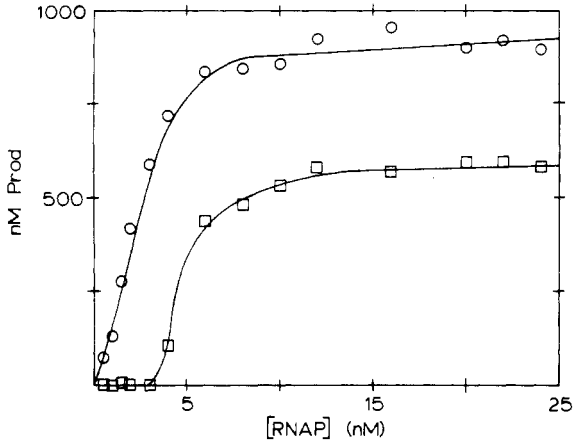


FIGURE 10: Competitive titration of A3 and E by abortive initiation. *Hinf*I$_{671}$ and *Hpa*–*Hae*$_{810}$ fragments were mixed, each at 1 nM, and preincubated 10 min with RNAP at concentrations indicated. Reactions were performed as described under Materials and Methods. Reaction aliquots were assayed for CpApU product 7.5 and 15 min after initiation for A3 (○) or for pApC product 10 and 20 min after initiation for E (□). No CpApU was produced from the *Hinf*I$_{671}$ (E) fragment, and no pApC was produced from the *Hpa*–*Hae*$_{810}$ (A3) fragment.

bp). Filter binding studies (Melancon et al., 1982) strongly suggested that the *Hae*III$_{716}$ fragment used in this work contained a tight-binding site capable of transcription.

Melancon et al. (1982) found that ATP plus UTP or ATP plus CTP stabilized the binding of RNAP to the *Hae*III$_{716}$ fragment in 0.6 M NaCl. Some stabilization of RNAP binding can occur during active abortive initiation even at 80 mM KCl (Cech & McClure, 1980; Prosen, 1985). The F promoter did support pApU and CpApU production as well as apparent synthesis of the expected tetramer with ATP plus UTP. On the other hand, essentially no abortive product was detected with ATP plus CTP or AMP plus CTP. There are sequences up to eight nucleotides in length on the *Hae*III$_{716}$ fragment that contain only A and C. An oligonucleotide five to eight nucleotides long produced at any of these sites would stabilize RNAP binding but have essentially no turnover. No promoter-like sequences were found near these A-C sequences, however.

Salt Dependence. The sensitivity of F promoter activity to ionic strength fits the general pattern observed for T7 binding sites. Mangel and Chamberlin (1974b) showed that increasing [KCl] from 0 to 100 mM had little or no effect on total transcriptional activity of T7, whereas from 100 to 200 mM KCl activity decreased 7-fold. Filter binding retention for a fragment containing the A1 and D promoters was virtually unchanged when [KCl] was increased from 40 to 80 mM but dropped 3-fold between 80 and 200 mM KCl (Strauss et al., 1980). Data in Figure 4 show little relative change in F promoter abortive-initiation turnover rate or runoff transcription from 0 to 100 mM KCl, although a maximum was observed at 80 mM. Abortive-initiation rates and runoff transcription decreased 6- and 10-fold, respectively, when [KCl] was increased from 100 to 200 mM. F promoter sensitivity also agrees reasonably well with the salt dependence observed in filter binding studies of T7 TB site-containing fragments (Melancon et al., 1982). At other promoters, abortive-initiation turnover was found to be more sensitive to ionic strength (T7 E and C, maximum turnover rate at 0 mM KCl, Prosen & Cech, 1985; λ b2 DNA, maximum at 25 mM KCl, McClure et al., 1978).

Kinetics. All evidence indicates that the unusual inhibition of F promoter activity at high [RNAP] is an intrinsic property of the promoter. Two different polymerase preparations produced identical results. Titrations of previously characterized T7 promoters with the same polymerase solutions (Figure 5) showed no inhibition. It is also unlikely that the inhibition of promoter activity observed in titration experiments can be indirectly accounted for by the reduction in the association rate. If a reaction already producing a steady-state turnover of product is subjected to a sudden increase in polymerase concentration, a new steady-state turnover corresponding to the new [RNAP] is achieved with a lag time of 0.5 min or less (final [RNAP] = 52 nM). This conclusion is also supported by the fact that identical titration profiles were obtained after preincubation times of 7.5 and 15 min (or 10 and 20 min in some experiments). We have no experimental evidence to support the mechanism proposed in Scheme II; independent reversible inhibition of RP$_c$ and RP$_o$ by a second polymerase molecule does, however, seem to provide the simplest explanation of the two observed inhibitory events (see below).

K_{I2}, as defined in Scheme II, can be reliably calculated from the *Hae*III$_{716}$ titration data (Figure 5) with eq 2. Other kinetic parameters can subsequently be estimated from *Hae*III$_{716}$ kinetic data (Figure 7) with nonlinear regression analysis and eq 1. An accurate fit of eq 1 is dependent on data obtained at RNAP concentrations below or at the onset of inhibition, i.e., less than 40 nM. Equation 1 is also derived by assuming polymerase is in excess (≥10 nM). We were unable to avoid a large scattering of data in this range. Calculated values of k_a, k_2, K_B, and K_{I1} are therefore only estimates. For eq 1 to describe data in Figure 7 where increasing [RNAP] results in decreased k_{obsd}, the $K_{I1}[R]^2$ term in the denominator must be much larger than the $K_{I2}[R]^2$ term in the numerator. In this respect, the estimated value for K_{I1} is at least qualitatively correct for F on the *Hae*III$_{716}$ fragment.

The k_a value (8.6×10^5 M^{-1} s^{-1}) estimated for the *Ava*II$_{498}$ data, corrected for inhibition, is less than the upper 67% confidence interval for k_a calculated without inhibition correction (9.2×10^5 M^{-1} s^{-1}). Consistency of the two approximations suggests that a reasonable k_a value for the F promoter is 9×10^5 M^{-1} s^{-1} or slightly less. (See discussion of competition experiments below for supportive evidence.) However, estimates of K_B and k_2 calculated with correction for inhibition deviate greatly from the respective values calculated without correction.

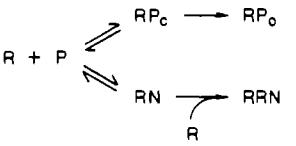
Kinetic data for the 498-bp fragment (Figure 7), which show little or no reduction of rates at high [RNAP], are fit by $K_{I1} = 1.6 \times 10^7$ M^{-1} (67% confidence interval between 3.8×10^7 and 3.9×10^6 M^{-1}). This represents a 100-fold reduction in the inhibitory binding of RNAP to RP$_i$ for the shorter 498-bp fragment compared to the 716-bp fragment. Since the *Ava*II$_{498}$ fragment is cut at position 66 (downstream) of the F promoter, the decrease in binding is possibly due to partial loss of an adjacent binding site. A potential secondary binding site is located at 32928 bp on the same strand as the F promoter. This site is intact on the 716-bp fragment but partially removed on the 498-bp fragment. The 32928 site has four mismatches with respect to the consensus sequence, a spacing of 16, and a very poor but potentially significant homology score (42.0) according to the method of Mulligan et al. (1984) (see below). The difficulty with this model is that the adjacent site must remain sufficiently intact on the shorter fragment to promote inhibitory binding to RP$_o$. Alternatively, a different mechanism, prehaps involving end binding, may be involved with similar results. The isomerization of RP$_i$ to RP$_o$ undoubtedly involves a conformational change in the protein

and/or the DNA, either of which could account for these two possibilities. A quite different model is one in which the binding of RNA polymerase to the F promoter facilitates the binding of a second RNA polymerase molecule to the first, i.e., dimerization mediated by a particular promoter sequence. Footprinting experiments may help determine the mechanism of inhibition. Preliminary experiments testing protection of nearby restriction sites after incubation with 50 mM RNAP have shown partial protection of an *Ava*II site 60 bp downstream from the 32988 site at a level 50% or less than that of *Mst*II protection within the F site (L. Zeiler and C. Cech, unpublished results).

Similar inhibition of the RNAP association rate has previously been reported (deHaseth et al., 1983) for RNAP binding to a synthetic 75-bp λ P_R promoter fragment. deHaseth et al. suggested that end binding was responsible, since no inhibition was observed with a much longer *Hae*III$_{890}$ λ P_R fragment. The model represented by Scheme II does not exclude end binding as the cause of adjacent inhibitory binding but does absolutely require the involvement of sequential binding by a second RNAP. Mutually exclusive binding of RNAP to either the promoter or an inhibitory site cannot explain the decrease in k_{obsd} observed upon increasing [RNAP] because such a scheme involves no $[R]^2$ terms. deHaseth et al. found that the observed association rate peaked at 20 nM RNAP then decreased, reaching an apparent plateau above 67 nM RNAP. According to Scheme II, a plateau should be observed that is proportional to the ratio K_{12}/K_{11}. We have also recently observed a similar but lesser degree of inhibition at the weak lac i^q promoter (P. Leith and C. Cech, unpublished results).

Shanblatt and Revzin (1984) observed an [RNAP]-dependent final extent of RP_o formation for the gal P2, lac UV5, and λ P_R promoters using a binding assay based on gel electrophoretic mobility of bound vs. unbound promoter-containing DNA fragments (235 bp, 203 and 1100 bp, and 890 bp long, respectively). Because complete titrations were not preformed, a direct comparison with the F promoter cannot be made. At least for the lac UV5 1100-bp fragment, however, a measurable reduction in open complex formation occurred at a significantly lower base pair per polymerase ratio than needed for F promoter inhibition. The gal P2 and lac UV5 promoters exhibited a reduction in association rate when the base pair/[RNAP] ratio was decreased from 20 to 4, and in addition, the kinetics of association appeared to be biphasic with an initial association too rapid to measure. The rate reduction observed by Shanblatt and Revzin was relieved by addition of nonspecific DNA. In our F promoter measurements, rate reduction (but not inhibition of final promoter occupancy) was relieved by using a smaller fragment cleaved near the promoter at one end. This had the net effect of reducing, not increasing, the amount of nonspecific DNA in the reaction. Moreover, the F promoter exhibited simpled pseudo-first-order kinetics of association.

Although quite different phenomena may be responsible for the differences in gal P2, lac UV5, and F promoter behavior, it is useful to consider the three potential inhibitory mechanisms proposed and analyzed by Shanblatt and Revzin. Mechanism I, an occlusion mechanism in which a competing binding site (proposed to be nonspecific) overlaps the promoter, is similar to the mechanism based on end binding proposed by deHaseth et al. (1983) for inhibition of the 75-bp synthetic λ P_R promoter. This mechanism predicts transcriptional inhiition but not rate reduction and does not predict an actual decrease in RP_o formation as [RNAP] increases. Mechanism II invokes the irreversible binding of a second polymerase molecule as a competing reaction for single polymerase binding:



This mechanism is most similar to the one we have proposed, and the equations derived from it have the same general form. It does not predict biphasic kinetics but explains the gal P2 and lac UV5 occupancy data qualitatively (not quantitatively.) However, we have not been able to fit our data to mechanism II equations with reasonable parameters. Moreover, F promoter inhibition is clearly reversible. Mechanism III is a bumping mechanism in which collisions between nonspecifically bound polymerase and the closed complex cause dissociation of polymerase from the promoter. This mechanism is attractive in that it accounts for gal P2 and lac UV5 biphasic kinetics. It unfortunately predicts 100% open complex formation independent of polymerase concentration. Such a mechanism should also be applicable to all promoters, at least weak promoters for which bumping prior to stable open complex formations is more probable. The λ P_{RM} promoter (Hawley & McClure, 1982; Shih & Gussin, 1983) exhibits normal monophasic association kinetics at 12 bp/RNAP on an 890-bp fragment. P_{RM} is equivalent to lac UV5 in K_B and considerably weaker in k_2. T7 minor promoters are similar to UV5 in both parameters (Dayton et al., 1984) but show no anamolous behavior. Although it is quite likely high polymerase concentrations cause some inhibition by a generalized mechanism at all promoters, we favor a model in which specific sequences at or near the promoter account for the more pronounced inhibition reported here.

Very likely there is a continuum of binding affinities of RNAP for DNA decreasing from very strong promoters to nonspecific binding (strong promoters > weak promoters > strong TB sites > weak TB sites > nonspecific binding) that will make straightforward classification of sites difficult. It has previously been shown that the minor C, D, and E promoters have k_a values 40–fold lower than the major A1 and A3 promoters (1×10^6 M^{-1} s^{-1} vs. $\geq 4 \times 10^7$ M^{-1} s^{-1}; Dayton et al., 1984; Prosen & Cech, 1985). Kadesch et al. (1980) have estimated the average second-order rate of association (k_a) for TB sites to be 1×10^5 M^{-1} s^{-1}. If, as generally assumed, the magnitude of k_a is directly proportional to promoter strength, the F promoter ($k_a \approx 9 \times 10^5$ M^{-1} s^{-1}) is essentially indistinguishable from the T7 minor promoters. Competitive titrations, on the other hand, show that the F site (perhaps at the upper extreme of TB site strength) is measurably weaker than the minor E promoter at low [RNAP], where inhibition ought to be insignificant. Probably our estimate of k_a is not quite accurate since it is, of course, dependent on the assumed model. At higher [RNAP] where inhibition alters the effective k_a, the important parameter is k_{obsd} at the concentration of polymerase in question. McClure et al. (1983) proposed that the effective RNAP concentration in vivo is 30 nM. At this concentration, k_{obsd} for the F promoter is about 2-fold less than k_{obsd} for the minor E promoter. The difference becomes 10-fold at very high [RNAP]. Substrate inhibition may at least account for the fact that in vitro transcription from this site has not been previously detected.

The exceptional agreement between the F promoter and consensus promoter sequences also suggests that the F promoter ought to be one of the strongest TB sites. Densitometric

analysis of filter binding studies on T7 *Hae*III fragments (Melancon et al., 1982) indeed showed that the *Hae*III$_{716}$ fragment bound RNAP better than other nonpromoter fragments of similar size. However, roughly 10 other nonpromoter *Hae*III fragments were retained on the filters nearly as well the 716-bp fragment. These fragments were stable to a 10-s heparin challenge after 15-min incubation at 37 °C in 100 mM KCl–10 mM $MgCl_2$ but not after only 0.5-min incubation, consistent with an association rate between 10^5 and 10^6 M^{-1} s^{-1} at the concentration of RNAP used. Titration of intact T7 (McClure et al., 1978) cannot be easily interpreted but does suggest the existence of relatively few sites on T7 that have a strong affinity for RNAP. (Intact phage λ DNA, on the other hand, exhibits titration behavior consistent with the presence of substantially more such sites.) If these sites of limited number are indeed similar in strength, they are characterized by association rates close to 10^6 M^{-1} s^{-1}. Kadesch et al. (1980) used electron microscopy to measure binding to the rightmost 10% of the T7 genome. About 15–20 sites were found on this fragment, characterized by an average association rate of 10^5 M^{-1} s^{-1}. These sites appeared to be transcriptionally inactive, although a quantitative analysis was not done. Kadesch et al. predicted about 100 such sites on whole T7. We suspect these sites belong to a different class with weaker affinity but larger number. Although it is thought that sequences matching the −10 region alone bind polymerase but cannot transcribe (West & Rodriguez, 1980), clearly not all TB sites fit this pattern. Our various searches for promoter-like sequences on T7 suggest that the large majority of these sites match both regions of the consensus sequence but in general have three to four mismatches that are more detrimental than those found in promoters.

We note that K_{eq} for RP_o formation, predicted from the measured kinetic parameters, is 6×10^8 M^{-1}. This in is excellent agreement with Melancon et al. (1982), who determined a value of 3×10^8 M^{-1} for 800-bp T7 fragments at low [RNAP]. Direct comparison is not possible because the 716-bp fragment probably binds polymerase somewhat tighter despite possible inhibition, solution conditions were not identical, and the necessary assumption of equilibrium in filter binding studies will not be quite accurate. The same order of magnitude, 10^9 M^{-1}, was observed in the electron microscopy work of Kadesch et al. (1980), although we have argued that these more numerous sites at the right end of T7 likely bind polymerase more slowly than does the F promoter.

We measured a lagtime of $t_{1/2} \approx 3$ min upon dilution of an ongoing reaction, reducing RNAP from 52 to 26 nM. For primary association of RNAP at 26 nM (18 nM active RNAP), $t_{1/2} = 1.4$ min would be expected. The difference between the observed lag and the minimum expected lag is significantly less than the observed dissociation rate of RNAP from the F promoter ($t_{1/2} = 8$ min). We therefore ignored the influence of inhibition during dissociation. We are uncertain whether the difference is small enough to assume binding of a second RNAP to RP_i or RP_o is in rapid equilibrium. It has been necessary to assume equilibrium for the analysis presented in this paper.

Dissociation of RNAP from the F promoter open complex ($t_{1/2} = 8$ min at 80 mM KCl) is in the same range as the reported average dissociation rate from TB sites ($t_{1/2} = 30$ min at 50 mM KCl and $t_{1/2} = 1$ min at 100 mM KCl; Kadesch et al., 1980). Possibly all TB sites dissociate rapidly but not more so than the minor E promoter ($t_{1/2} = 13$ min at 80 mM KCl; Prosen & Cech, 1985). These dissociation rates are still much slower than the forward rate of isomerization into RP_o and faster than initiation of transcription for other T7 promoters (Mangel & Chamberlin, 1974a). Unless future experiments uncover a rate-limiting step subsequent to RP_o formation, in vivo utilization of the F promoter is likely to be solely a function of the kinetics of association.

As discussed, the competition experiment between E and F (Figure 9) showed that the E promoter is somewhat stronger than the F promoter (716-bp fragment) at low RNAP concentrations. A similar competition experiment between A3 and E (Figure 10) showed that the A3 promoter is much stronger than the E promoter. In the latter case, the results directly correlate with the magnitude of measured association rates.

Sequence Comparisons. The −35 regions of the A3 and F promoters are identical with the consensus sequence, and both promoters have the optimal spacing of 17 bp (Figure 1). Only the −10 regions differ from the consensus. The F promoter has a cytidine residue at −13 rather than the normal thymidine. Of 112 compiled promoter sequences (Hawley & McClure, 1983), only 10 promoters including the weak λ P_o and the very weak constitutive lac i promoter have a cytidine in this position. According to the sequence rating method of Mulligan et al. (1984), where a homology score >30 may indicate transcriptional activity, the F promoter (homology score = 63.9) should be much weaker than the A3 promoter (homology score = 72.8), as strong as the T7 D promoter (homology score = 64.0), and stronger than the E promoter (homology score = 56.2). The F promoter is indeed much weaker than the A3 promoter in vitro but competes less effectively for limiting [RNAP] than either D or E. However, the measured k_a values for these three promoters all fall within 1 SD of the line correlating k_a with homology score.

Summary. Characterization of a bacteriophage T7 tight binding site that is fully active transcriptionally in vitro has been established. This site, referred to as the F promoter, is much weaker than the major A3 and slightly weaker than the minor E promoter. It appears to be located adjacent to an even weaker RNAP binding site that causes substrate-like inhibition of F promoter function at high [RNAP]. Whether RNAP inhibition plays an in vivo role is uncertain. Nevertheless, it is clear that when measuring promoter activity in vitro, the possibility of interference from adjacent sites or ends must be considered.

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