THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1984 by The American Society of Biological Chemists, Inc.

Vol. 259, No. 3, Issue of February 10, pp. 1616–1621, 1984
Printed in U.S.A.

Kinetic Measurements of *Escherichia coli* RNA Polymerase Association with Bacteriophage T7 Early Promoters*

(Received for publication, April 15, 1983)

Christopher J. Dayton, Dennis E. Prosen‡, Kathryn L. Parker, and Carol L. Cech§

From the Department of Chemistry, University of Colorado, Boulder, Colorado 80309

During infection of *Escherichia coli* by bacteriophage T7, *E. coli* RNA polymerase utilizes only three promoters (A1, A2, and A3). *In vitro*, the A promoters predominate at very low polymerase concentration, but at higher polymerase concentration the minor B, C, D, and E promoters are used with equal efficiency. The binding constant for the initial association of polymerase with promoters and the forward rate of isomerization to an "open" complex capable of initiation have been measured for the A1, A3, C, and D promoters using the abortive initiation reaction. At 80 mM KCl, 37 °C, both major and minor promoters isomerize rapidly ($t_{1/2}$ = 10 to 30 s). In contrast, initial binding to the minor promoters ($K_I = 10^7$) is at least 10-fold weaker than binding to major promoters ($K_I \geq 10^8$), suggesting promoter selectivity in the T7 system occurs at the point of initial binding. Association kinetics of the A1 and C promoters on intact T7 were the same as measured on restriction fragments of length ≥500 base pairs. All open complexes dissociated with half-lives longer than 1 h. Overall equilibrium binding constants estimated from kinetic measurements ranged from 10^{10} to $\geq 10^{11}$ M^{-1} for minor and major promoters, respectively. Data on heparin attack and abortive initiation turnover rates indicate open complex polymerase conformation may be different at the A1 and A3 promoters.

Selective interaction of RNA polymerase with promoter regions of DNA is a major control point for regulation of cell growth in prokaryotes (reviewed in Refs. 1–3). Initiation of transcription at some promoters is modulated by the presence or absence of effector molecules, but for many well-studied *Escherichia coli* promoters the sole determinant of the frequency of initiation appears to be the association of the promoter with RNA polymerase. Bacteriophage T7 is a standard example. Early in infection, *E. coli* RNA polymerase initiates at roughly equal frequency from the closely spaced A1, A2, and A3 promoters (Fig. 1). A product of this message is a T7-specific RNA polymerase responsible for middle and late T7 transcription. No other *E. coli* RNA polymerase-specific transcripts have been detected in significant amounts *in vivo* (4–6). At very low polymerase concentration, the "major" A promoters predominate *in vitro* as well. At higher (but less than saturating) polymerase concentration, however, the "minor" B, C, D, and E promoters transcribe with equal efficiency (7–9). Thus, the differences in polymerase association and initiation at minor compared to major T7 promoters might be expected to be small but critical in determining function *in vivo*.

The extent of repression of the lac operon by lac repressor correlates directly with the extent of equilibrium repressor-operator binding (10). In the case of RNA polymerase and T7 promoters, however, dissociation of the polymerase-promoter complex is extremely slow (9) relative to the rate of initiation (1, 11, 12), such that equilibrium of polymerase with promoter is not obtained before initiation of RNA synthesis. The molecular mechanism controlling promoter selectivity must depend on the kinetics of association. In this paper, we present detailed measurements of the kinetics of polymerase interactions with two major (A1 and A3) and two minor (C and D) T7 promoters contained on isolated T7 restriction fragments. Measurements were done under solution conditions for which we also made comparative promoter utilization studies.[1]

A minimum two-step association of polymerase with the promoter (13) is well established (1, 14, 15). It may be schematically written as

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

where RP_i represents the initial site-specific complex of RNA polymerase (R) with promoter (P), and RP_o represents the final complex primed for initiation upon addition of nucleoside triphosphates. RP_o is generally referred to as the "open complex" since base pairing near the initiation point has been disrupted (16, 17). We will not use the common term "closed complex" for RP_i, since kinetically any number of closed intermediates may occur between RP_i and RP_o. Stahl and Chamberlin (7) presented data suggesting that both major and minor T7 promoters rapidly form initial binding complexes but that discrimination occurs at the second step in favor of major promoters. Until recently, no available technique provided for direct measurement of these individual steps. McClure (14) has now shown that the delay (τ) in approach to steady state turnover of abortive initiation product after mixing of polymerase and promoter in the presence of nucleoside triphosphates may be used to determine the initial binding constant $K_I = k_1/k_{-1}$ and the isomerization rate k_2. A related method employing full-length transcription yields similar data (15). We have applied the abortive initiation approach to the T7 promoters since quantitation is more facile. We have also measured k_{-2}, and overall equilibrium

* This investigation was supported by Grant MV-140 from the American Cancer Society, Public Health Service Grant AI14938, a grant from The Jane Coffin Childs Memorial Fund for Medical Research, and a Biomedical Science Support Grant to the University of Colorado.

‡ Supported in part as a trainee on Public Health Service Grant GM007135.

§ To whom correspondence should be addressed at, Department of Chemistry, Campus Box 215, University of Colorado, Boulder, CO 80309.

[1] C. J. Dayton, D. E. Prosen, K. L. Parker, and C. L. Cech, manuscript in preparation.

binding constants relative to synthetic copolymers, by the methods of Cech and McClure (9).

MATERIALS AND METHODS

Polymerase and DNA—RNA polymerase was isolated from *E. coli* (Grain Processing) by the method of Burgess and Jendrisak (18). Per cent active polymerase was measured periodically by the T7 template functional assay method of Chamberlin *et al.* (19). 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 (9). Poly(dAT)[2] and poly(dIC) were purchased (P-L Biochemicals). Nucleoside triphosphates (P-L Biochemicals) were purified as previously described (11). Fragments of DNA created by restriction nuclease digestion were isolated after gel electrophoresis by electroelution from gel strips or by digestion of *N,N'*-bis-acrylylcystamine acrylamide gels with 2-mercaptoethanol (20). Contaminating acrylamide was removed by loading DNA onto DEAE-A25 Sephadex columns, rinsing with 0.1 M KCl, and eluting the DNA at 1 M KCl. DNA was then dialyzed into 0.05 M KCl, 0.01 M Tris (pH 7.5), 0.001 M EDTA. Promoter-containing fragments were identified using published gel patterns (21–24) and sequence data for the left end of T7 (25). The fragments used in this work are illustrated in Fig. 1. T7D111 is a deletion mutant missing A2 and A3 (total deletion is 1131 base pairs[3]). T7C5 is functionally identical to wild type but is used because the left-end *Hae*III fragment runs free from contaminating fragments on preparative gels.

Abortive Initiation Assays—The principle and properties of the abortive initiation assay have been reported (26, 27). Briefly, abortive initiation is the reiterative production of a short oligoribonucleotide, usually a dinucleoside tetraphosphate, when only two or three nucleoside triphosphates are present in a transcription reaction. The abortive initiation products used in this work were pppApU for the A1 and A3 promoters, pGpUpU for the D promoter, and pApC for the C promoter. Product was separated as described (11) by ascending chromatography.

To obtain association delay times (τ), the appropriate combination of starting nucleotide (1 mM) and elongating nucleotide (0.04 mM), with the corresponding α-^{32}P-labeled nucleotide added to between 300 and 2000 cpm/pmol, was preincubated 10 min at 37 °C with DNA template (2 nM genome) in standard reaction buffer (80 mM KCl, 40 mM Tris, pH 8, 10 mM $MgCl_2$, and 1 mM dithiothreitol.) RNA polymerase at concentrations varying from 25 to 250 nM was added at time zero. Aliquots (10 μl) were taken at appropriate times and streaked on paper prestreaked with 0.1 M EDTA to assure quenching of the reaction. Control reactions were identical except that RNA polymerase was preincubated 10 min with DNA and the reaction initiated by addition of nucleotides in reaction buffer. The delay time, τ, was determined by nonlinear regression analysis of the rate of approach to linear abortive initiation turnover. Assuming that both k_1 and k_{-1} are fast relative to k_2 (pre-equilibrium condition), that $k_{-2} \ll k_2$ (see Ref. 9 for supporting arguments), that polymerase is in excess, and $d(RP_i)/dt = 0$, then τ reduces to

$$\tau = \frac{1}{k_2} + \frac{k_{-1}}{k_1 k_2 (R)}$$

(14). A plot of τ *versus* $1/R$ is referred to by McClure (14) as a TAU plot. The τ intercept yields $1/k_2$ directly; the inverse of the slope corrected for k_2 yields $K_I = k_1/k_{-1}$. TAU plots were constructed using estimated active polymerase concentrations since the slope depends on $[R]$.

To obtain dissociation rates and relative equilibrium binding constants, the methods described by Cech and McClure (9) were followed. Briefly, dissociation was measured by preincubating 40 nM polymerase with 2 nM template for 10 min at 37 °C prior to addition of competing synthetic copolymer DNA (0.05 mM phosphate for poly(dAT), 1 mM phosphate for poly(dIC)) at time zero. Aliquots were removed at later times and mixed with nucleotides in reaction buffer for 5 to 10 min, depending on the stability of the complex. Relative binding constants were obtained by first incubating polymerase with nonsaturating concentrations of synthetic copolymer (on the order of 20 μM poly(dAT), 200 μM poly(dIC)) 5 min at 37 °C before addition of 2 nM promoter-containing template. At times ranging from 5 min to 2 h later, aliquots were removed and mixed with appropriate nucleotides to monitor abortive initiation for 10 min. Turnover rates independent of time of incubation were taken as indication that equilibrium between promoter and copolymer binding had been achieved. Relative binding constants were calculated from equilibrium turnover rates relative to maximum turnover rates at full promoter occupancy using the theory of McGhee and von Hippel (28) to correct for overlapping site effects.

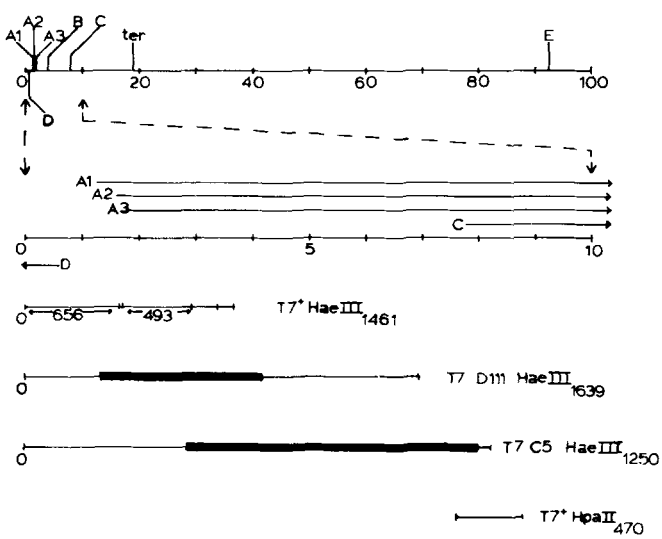

FIG. 1. **Transcriptional map of T7 for *E. coli* RNA polymerase.** "A" promoters are active *in vivo* early in infection. B, C, D, and E promoters are known to transcribe only *in vitro*. Restriction fragments used in this work are also shown. Lengths of fragments and positions in gel patterns were determined from Refs. 21–25.

RESULTS

From the slope and intercept of TAU plots (see "Materials and Methods") values of k_2 and K_I were determined for promoters on T7 restriction fragments. Table I is a summary of data measured at 37 °C under standard conditions for the following promoters: A1 and D promoters on the T7D111 left-end *Hae*III$_{1639}$ fragment; the A3 promoter on the T7$^+$ *Hha*I$_{493}$ fragment; and the C promoter on the T7D111 *Hpa*II$_{470}$ fragment. All reported data for k_a and K_I are based on estimated active polymerase concentrations. A typical lagtime (τ) experiment for the D promoter is plotted in Fig. 2. τ *versus* $1/R$ (TAU plot) is shown for the A1, C, and D promoters in Fig. 3. We estimate error in τ to be 10–20% or a minimum ±10 s. This means that the relative error for the rapidly associating A promoters is rather high. The slope of TAU plots, from which K_I is derived, is subject to an additional error of 10–20% in estimates of polymerase activity. The standard deviation escalates for the major promoters since the slope is so small relative to errors in τ. For the major promoters, k_2 is accurate within a factor of 2 or less, but K_I represents an approximation, perhaps only a lower limit.

Given the condition $k_{-1} \gg k_2$, k_{-2} equals the dissociation rate, k_{off}, observed when RP_o is challenged with a sequestering agent for free polymerase (14). Experimental procedure has been described under "Materials and Methods." Table I contains k_{-2} data measured for the A1 and A3 promoters using poly(dIC) as challenger (see also Fig. 4) and for the C promoter using poly(dAT). Dissociation from the D promoter was measured with both synthetic copolymers, since the promoter-specific abortive initiation product pGpUpU was not synthesized from either copolymer alone. Identical results were obtained with both challenging agents. Results were inde-

[2] The abbreviations used are: poly(dAT), poly[d(A-T):d(A-T)]; poly(dIC), poly[d(I-C):d(I-C)].

[3] W. Studier, personal communication.

TABLE I

Kinetic parameters of major (A) and minor (C and D) T7 promoters

Data determined as described under "Materials and Methods," at 37 °C and 80 mM KCl unless otherwise indicated.

	A1	A3	D	C	C[a]	D[b]
Intercept of TAU plot (s)[c]	11	24	17	27	43	28
$k_2(s^{-1})$	0.09	0.04	0.06	0.04	0.02	0.04
k_a = 1/slope of TAU plot (M^{-1} s^{-1})	3×10^7	2×10^7	1×10^6	1×10^6	5×10^4	1×10^6
K_I (M^{-1})[d]	3×10^8	4×10^8	2×10^7	3×10^7	2×10^6	4×10^7
$t_{1/2}$ for k_d (min)	64	64	230	200	68	360
$k_{-2}(s^{-1})$[e]	2×10^{-4}	2×10^{-4}	5×10^{-5}	6×10^{-5}	2×10^{-4}	3×10^{-5}
$K_{eq} = K_I k_2/k_{-2}$ (predicted, M^{-1})	1×10^{11}	1×10^{11}	2×10^{10}	2×10^{10}	3×10^8	4×10^{10}

[a] Measured at 10 °C, 80 mM KCl.
[b] Measured at 37 °C, 100 mM KCl.
[c] Estimated uncertainty ±10 s.
[d] Estimated uncertainty ±50%, lower limit for A promoters.
[e] Estimated uncertainty ±30%.

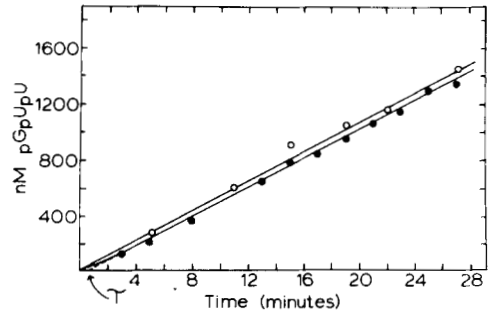

FIG. 2. **Measurement of delay time τ for T7 D promoter at 89 nM active RNA polymerase.** Reaction mixture contained 2 nM T7D111 *Hae*III$_{1639}$ fragment, 80 mM KCl, 40 mM Tris, pH 8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM GMP, and 0.04 mM [α-^{32}P]UTP. O——O, RNA polymerase was preincubated with template at 37 °C 10 min before addition of GMP + UTP at time zero. Total accumulated product pGpUpU as a function of time is plotted. ●——●, template was incubated with GMP + UTP for 10 min before addition of RNA polymerase at time zero. The determination of $\tau = 1/k_{obs}$ assumes $[RP_o] = [P_T](1 - e^{-k_{obs}t})$.

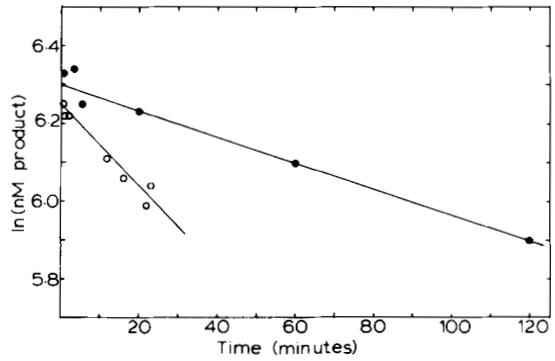

FIG. 4. **Dissociation rates, $k_d = k_{-2}$, determined by excess copolymer challenge for the A1 promoter on the T7D111 *Hae*III$_{1639}$ fragment using 1 mM (phosphate) poly(dIC) (O——O); and the C promoter on the T7$^+$ *Hpa*II$_{470}$ fragment using 50 μM (phosphate) poly(dAT) (●——●).** Promoter (2 nM) was preincubated under standard conditions 10 min at 37 °C before addition of copolymer at time zero. Aliquots were taken at indicated times and assayed for abortive initiation product accumulated in 5 min as described under "Materials and Methods."

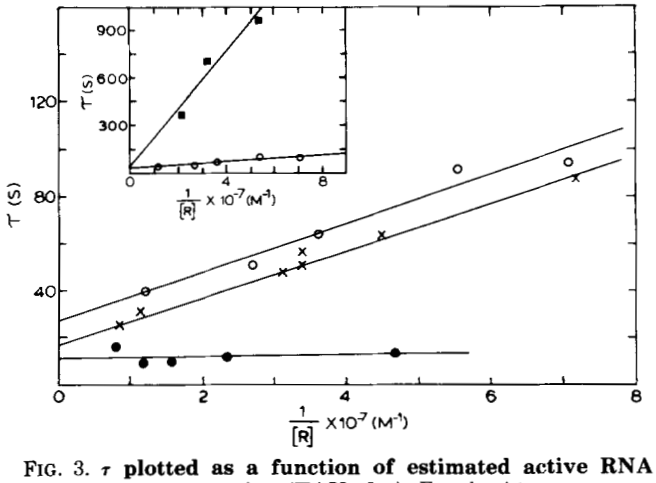

FIG. 3. **τ plotted as a function of estimated active RNA polymerase concentration (TAU plot).** For the A1 promoter on the T7D111 *Hae*III$_{1639}$ fragment at 37 °C (●——●); for the D promoter (same fragment) at 37 °C (×——×); for the C promoter on the T7$^+$ *Hpa*II$_{470}$ fragment at 37 °C (O——O); and for the C promoter (same fragment) at 10 °C (■——■). Experimental protocol is as described in Fig. 2.

pendent of copolymer concentration in the range tested for these experiments (0.5 to 1 mM poly(dAT), 0.8 to 2 mM poly(dIC).

Equilibrium binding constants $K_{eq} = k_1 k_2/k_{-1}k_{-2}$, calculated from measured kinetic parameters, are on the order of 10^{11} and 10^{10} M^{-1} for the major and minor promoters, respectively (Table I). Direct measurement of these constants has been difficult, in part because the binding constant is so high. Distribution of polymerase between promoter and a synthetic copolymer is, however, easy to quantitate by abortive initiation. Moreover, if polymerase is first bound to synthetic copolymer before addition of promoter-containing DNA, an equilibrium distribution is rapidly achieved. This procedure, using abortive initiation to determine per cent polymerase bound to promoter *versus* poly(dAT) or poly(dIC), was used to determine $K_{R \cdot \text{promoter}}/K_{R \cdot \text{copolymer}}$ (see "Materials and Methods"). Binding of polymerase to the A1 promoter relative to poly(dAT) as well as to poly(dIC) was measured using CpA to prime CpApU synthesis from the promoter. Poly(dAT) was found to support no synthesis of CpApU. Thus, the ratio $K_{R \cdot dAT}/K_{R \cdot dIC} \cong 6$ was established, making possible comparison of binding constants for any two procedures. Ratios determined by comparison of relative binding constants are listed in Table II.

In order to compare our results with related published

TABLE II

Relative binding constants

Estimated uncertainty ±50%.

Measured	Predicted from kinetic constants
$K_{R\cdot A1}/K_{R\cdot dAT} = 80$	$K_{R\cdot A1}/K_{R\cdot D} = 5$
$K_{R\cdot A1}/K_{R\cdot dIC} = 500$	$K_{R\cdot D}/K_{R\cdot C} = 1$
$K_{R\cdot D}/K_{R\cdot dAT} = 20$	
$K_{R\cdot C}/K_{R\cdot dAT} = 24$	

observations, two variations in salt and temperature were tested. Association and dissociation parameters were measured for the D promoter at 100 mM KCl and for the C promoter at 10 °C. The increase in KCl concentration to 100 mM reproduced the conditions under which McClure (14) examined the D promoter on intact T7 template. Using the same *Hae*III$_{1650}$ fragment as in the 80 mM studies, we found that k_2 decreased from 0.06 to 0.04 s^{-1}, and K_I increased from 2×10^7 to 4×10^7 M^{-1} (Table I). We chose to test the C promoter at 10 °C, because Wiggs *et al.* (8) reported very poor utilization of this promoter at low temperature. The drop in temperature surprisingly reduced the value of k_2 for the C promoter by only a factor of 2, but decreased K_I by an order of magnitude (Table I).

We also measured association rates at 80 mM KCl for the A1 and C promoters on intact T7D111 DNA. We could detect no significant difference, over a wide range of polymerase concentration, from measurements on 1639 and 470 base pair fragments, respectively (data not shown).

Previous attempts to compare abortive initiation turnover rates from promoters that direct synthesis of the same product have been inconclusive (27). A major difficulty has been uncertainty in template concentration due to contaminating DNA fragments. We circumvented this problem by isolating a 656 base pair fragment containing A1, and a 493 base pair fragment containing A3, by secondary *Hha*I digestion of a previously isolated T7$^+$ *Hae*III left-end fragment (Fig. 1). Gel analysis showed no contamination. To confirm template concentration calculated from UV absorption, both fragments were independently titrated with RNA polymerase. Maximum pppApU production occurred at one active polymerase/promoter for each fragment. Abortive initiation turnover was then assayed under identical conditions on the two fragments. The amount of pppApU produced per min/genome averaged 65 for the A1 promoter and 20 for the A3 promoter. Using the T7C5 *Hae*III$_{1250}$ fragment that contains both promoters, turnover was roughly 130 (less accurate due to DNA concentration uncertainty). We conclude that the abortive initiation turnover rate is promoter-dependent even when the product dinucleotide is the same for both promoters. This result was confirmed by measuring residual pppApU production from the *Hae*III$_{1250}$ fragment after cleavage of the DNA within either the A1 or A3 promoter sequence by an appropriate restriction nuclease (*Taq*I for inactivation of A1, *Hin*fI for inactivation of A3). Although this procedure was less quantitative due to a slight inhibition of the turnover rate by the presence of restriction nucleases, the same approximate ratio of turnover rates for A1 *versus* A3 was observed. Cleavage by both restriction nucleases did not entirely eliminate abortive initiation product accumulation. The most probable explanation is that other sites on the intact left-end fragment produce small amounts of pppApU.

DISCUSSION

The A1 and A3 promoters are both characterized by strong initial equilibrium association with polymerase ($K_I \cong 4 \times 10^8$) and rapid conversion to the RP_o complex (half-life in the range of 10 to 30 s). McClure (14) reported very similar data for the A2 promoter measured on intact T7C5 DNA. The overall rate of association, $k_a = K_I k_2$, is slightly faster than reported rates for λ P_R (29) and lacUV5 (30). Note that K_I may very well be larger than estimated, since the extremely weak dependence on polymerase concentration coupled with estimated error in the data does not allow for determination of an upper limit. Both promoters exhibit a dissociation half-life of about 60 min. Predicted binding constants for both promoters are therefore $\geq 10^{11}$ M^{-1}. The similarity of the measured parameters for the three A promoters is not surprising, since no significant differences in utilization have been observed (9). Rosenberg *et al.* (31) reported a very similar overall rate of open complex formation for A1 on an intact T7 template measured by what they term the quantitative transcription assay. However, the same group found the half-life of dissociation for the A1 open complex to be only 5 min at 100 mM NaCl (32).

Polymerase association with each of the two minor promoters, C and D, is also nearly identical kinetically. We found that the minor promoters undergo a relatively rapid isomerization to the open complex only 2- or 3-fold slower than the fastest A promoter. The range of k_2 values for the major promoters indeed overlaps that of the minor promoters. More striking is the 10-fold lower initial binding constant K_I of the minor promoters. Two conclusions may be derived. First, the fact that measured values are highly dependent on the strength of the promoter but not dependent on the size of the DNA template (in the range studied) reinforces the assumption that measured K_I values reflect promoter-specific binding. Second, the fact that relative differences between major and minor promoters are much larger for K_I than for k_2 suggests that initial binding rather than the rate of isomerization may control the pattern of promoter utilization during early T7 infection. A similar pattern was observed for the λ P_R promoter compared to the x3 mutant, although in this case the relative differences in both parameters were larger (19). The T7 minor promoters actually form open complexes somewhat faster than does the extremely weak λ P_{RM} promoter in the presence of cI repressor (33, 34).

Stefano and Gralla (30) have pointed out that when the RP_i complex is sufficiently unstable, the observed K_I becomes a measure of nK_B, where n is the number of nonspecific binding sites overlapping the promoter and K_B is the nonspecific association binding constant. Since a reliable value for K_B has not been established, it is unclear whether the K_I values for the minor promoters represent the true values or rather an upper limit. Published K_I data for the λ P_R–x3 mutation (29), λ P_{RM} (33, 34), and 7 *lac* promoter mutations (30) all fall between 0.6×10^7 and 3×10^7 M^{-1}. No lower values have been reported. The overall association rate $k_a = K_I k_2$ is, however, unaffected by competition from nonspecific binding (30).

Sequences for the A1, A2, A3 (23), C (35), and D (25) promoters along with a composite promoter of most frequently observed sequence (2, 36) are shown in Fig. 5. Insufficient information is available to determine what sequence characteristics distinguish major from minor promoters in the T7 system. The T7 promoters in fact match the canonical promoter sequence remarkably well in comparison to other promoter sequences which are known to be used *in vivo* (2, 33). Considering only the 6 base pair −10 and −35 regions and the size of the spacer region, the minor promoter sequences in general contain only one additional mismatch than do the major promoters. The sequence of the minor D promoter, for

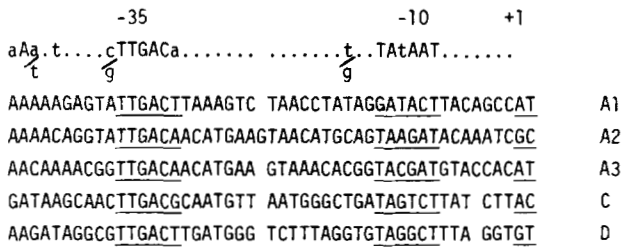

FIG. 5. **T7 early promoter sequences taken from references 23 (*A1, A2,* and *A3*), 35 (*C*), and 25 (*D*).** The most frequently observed bases (2, 36) are shown for comparison. *Capital letters* represent bases present in more than 61% of sequenced promoters. *Lower case* letters represent a frequency ≥46%.

example, is identical to that of the major A1 promoter in the −35 region and in the size of the spacer. All bases of the −10 region of the D promoter occur in the same position at least once in one of the A promoters, except the G at position −11 which occurs rarely in known promoters. Other less obvious differences outside the 12 base pairs of the −10 and −35 regions may of course contribute significantly to the assumed inactivity of the minor promoters *in vivo*. In particular, the region of lesser homology at −45 may be important (36). Nevertheless, the T7 promoter characteristics appear to refute the concept that the sequence of the −10 region controls the rate of isomerization but not initial binding.

Tau plot data for the D promoter at 100 mM KCl measured on the *Hae*III$_{1639}$ fragment is in good agreement with the data for the D promoter on intact T7 DNA published by McClure (14). We used estimated active polymerase concentrations to determine K_I values. If the slope of McClure's data is corrected for his estimate of polymerase activity, agreement is within experimental error. These data along with our own observations on the effect of template length on A1 and C promoter behavior suggest that at 80 to 100 mM KCl, polymerase association kinetics are independent of template length for length ≥ 500 base pairs.

The C promotor was chosen for testing at 10 °C because of evidence that it is used very infrequently, if at all, in comparison to other T7 promoters at low temperature *in vitro* (8). Fortunately, abortive initiation turnover was substantial at 10 °C if preincubation was sufficiently long. Tau plot analysis surprisingly showed little change in the isomerization rate but rather a large decrease in the initial binding constant. The low temperature K_I is in fact an order of magnitude lower than the minimum observed at 37 °C (see above). Both the low magnitude and the unexpected temperature dependence of K_I might be explained by temperature dependent nonspecific binding, but the data of de Haseth *et al.* (37) do not support this hypothesis. Presumption of a 3-step mechanism with the first two steps in pre-equilibrium (38) is of limited help. The decrease in K_I observed at low temperature may be assumed to be entirely in the second pre-equilibrium step, perhaps involving a protein conformational change. However, the maximum reduction in the true value of k_2 at low temperature would still be only a factor of 2.

Dissociation rates for the A1, A2, A3, and D promoters determined by heparin challenge and for the A2 and D promoters measured by poly(dAT) challenge were reported earlier (9). We have found that poly(dIC) functions as well as poly(dAT) as challenging agent, although the concentration which must be used to sequester all free polymerase is approximately 10-fold higher. Dissociation rates clearly bear no direct relationship to promoter strength. Of the T7 promoters that have been studied, the minor promoters have significantly slower rates of dissociation than do the major A promoters. Neither can a direct inverse relationship exist, because the strong λ P_R promoter and its mutant x3 have identical dissociation rates of the same magnitude as the T7 minor promoters (29). In all cases, however, two necessary assumptions, that dissociation is very slow relative to the forward rate of isomerization and to the time required for determining τ, are valid.

Heparin has frequently been used as a sequestering agent for dissociation assays despite evidence that it directly attacks polymerase-promoter complexes (9, 39). Using heparin, Cech and McClure (9) reported dissociation half-lives of about 5, 30, 50, and 210 min for the A1, A2, A3, and D promoters, respectively, at heparin concentrations ≤80 μg/ml. For all but the A1 promoter, these rates satisfactorily match those reported here and by Cech and McClure (9) from copolymer challenge experiments. Cech and McClure also reported the rate of direct attack of heparin on the polymerase-D promoter complex to be 340 M^{-1} min^{-1}. If the same attack rate applied to the A1 promoter complex, the expected half-life extrapolated to zero heparin concentration would increase from 5 to 5.03 min, not 60 min as measured by either poly(dAT) or poly(dIC)challenge. Pfeffer *et al.* (39) also observed an apparent high degree of heparin destabilization of the A1 complex. Our data indicate that the A1 promoter is unique in its extreme sensitivity to heparin. This sensitivity may be due to a unique conformation of the open complex at the A1 promoter. In any case, it must be concluded that heparin challenge is not a reliable technique for application to all promoters.

Absolute equilibrium binding constants have not been directly determined. Nevertheless, it is possible to compare measured ratios to ratios calculated from measured rate constants. Agreement of the predicted and observed ratios listed in Table I and II is good. The magnitude of the predicted absolute binding constants is also within the range expected from competition filter binding assays (40). Using a template competition assay, Kadesch *et al.* (41) estimated $K_{eq} = 6 \times 10^{11}$ M^{-1} for the A1 promoter at 100 mM NaCl. The advantage of using kinetic measurements to establish equilibrium constants is that the question of whether or not equilibrium has indeed been attained need not be raised.

The initiation step itself, schematically identified by the rate constant k_3, must also be considered a potential regulatory step. The rate of formation of the first few phosphodiester bonds may be promoter-dependent, or natural abortive initiation (recycling) even in the presence of physiological concentrations of all four nucleotides may reduce the rate of competent RNA synthesis. Natural abortive initiation of products two to eight bases in length is now known to occur with high frequency at the lacUV5 (42) and Tn5 (43) promoters, and probably with lower frequency at λ P_R, (44). In the first two cases, recycling may very well be rate-limiting *in vivo*. However, no evidence exists for natural abortive initiation at the T7 A promoters (45). Less is known about relative rates of formation of initial phosphodiester bonds. It is not yet clear how the steady state rate of abortive initiation should be interpreted in relation to the rate of initial phosphodiester bond formation during productive RNA synthesis. It has been argued that the release of the abortive initiation product can at most be only partially rate-limiting during steady state abortive initiation (11). Comparison of abortive initiation turnover rates for pppApU from the A1 and A3 promoters therefore suggests that the rate of formation of a bond of identical sequence may be promoter-specific. Barring stabilization of the ternary complex by adjacent unpaired DNA bases, the abortive initiation rate probably reflects promoter-

specific differences in open complex conformation. It is entirely possible such differences in rates of bond formation are never large enough to influence the overall rate of mRNA initiation and synthesis (11, 27, 45), but it should be kept in mind that the potential exists for promoter-dependent rates of phosphodiester bond formation.

In summary, the ability to measure the kinetics of RNA polymerase-DNA interactions is a necessary step in the general effort to correlate the sequence of DNA regulatory regions with control of gene expression. Data presented here demonstrate that the rate of association of polymerase with promoter and conversion to the RP_o complex is greater than 10-fold faster for the very strong T7 major promoters than for the less strong minor promoters. The latter are apparently not used *in vivo* but are very active during *in vitro* transcription at polymerase concentrations far below saturation levels for the major promoters. No other transcriptional property of these promoters can be correlated with frequency of utilization. Nearly all of this difference in k_a resides in the initial binding step $R + P \rightleftharpoons RP_i$. Speculation at this point as to what features of promoter sequence distinguish the major from minor promoters is premature. Only more data on the effects of mutations or stepwise chemical changes in promoter sequence can resolve this question.

Acknowledgments—The expert technical assistance of Cheryl A. Ristig is gratefully acknowledged. Our program for non-linear regression analysis was based on a program kindly provided by William R. McClure.

REFERENCES

1. Chamberlin, M. J. (1976) in *RNA Polymerase* (Losick, R., and Chamberlin, M., eds) pp. 17–67 and 159–191, Cold Spring Harbor Laboratory, New York
2. Rosenberg, M., and Court, D. (1979) *Annu. Rev. Genet.* **13,** 319–353
3. Pribnow, D. (1979) in *Biological Regulation and Development* (Goldberger, R. F., ed) Vol 1, pp. 219–269, Plenum Press, New York
4. Dunn, J., and Studier, W. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 3296–3300
5. Kramer, R., Rosenberg, M., and Steitz, J. (1974) *J. Mol. Biol.* **89,** 767–776
6. Hercules, K., Javanovich, S., and Sauerbier, W. (1976) *J. Virol.* **17,** 642–658
7. Stahl, S. J., and Chamberlin, M. J. (1977) *J. Mol. Biol.* **112,** 577–601
8. Wiggs, J. L., Bush, J. W., and Chamberlin, M. J. (1979) *Cell* **16,** 97–109
9. Cech, C. L., and McClure, W. R. (1980) *Biochemistry* **19,** 2440–2447
10. von Hippel, P. (1979) in *Biological Regulation and Development* (Goldberger, R. F., ed) Vol 1, pp. 279–347, Plenum Press, New York
11. McClure, W. R., Cech, C. L., and Johnston, D. E. (1978) *J. Biol. Chem.* **253,** 8941–8948
12. Nierman, W. C., and Chamberlin, M. J. (1979) *J. Biol. Chem.* **254,** 7921–7926
13. Walter, G., Zillig, W., Palm, P., and Fuchs, E. (1967) *Eur. J. Biochem.* **3,** 194–201
14. McClure, W. R. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5634–5638.
15. Stefano, J. E., and Gralla, J. D. (1980) *J. Biol. Chem.* **255,** 10423–10430
16. Hsieh, T., and Wang, J. C. (1978) *Nucleic Acids Res.* **5,** 3337–3345
17. Siebenlist, U. (1979) *Nature (Lond.)* **279,** 651–652
18. Burgess, R. R., and Jendrisak, J. J. (1975) *Biochemistry* **14,** 4634–4638
19. Chamberlin, M. J., Nierman, W. C., Wiggs, J., and Neff, N. (1979) *J. Biol. Chem.* **254,** 10061–10069
20. Hansen, J. N. (1977) *Anal. Biochem.* **76,** 37–44
21. Hsieh, T., and Wang, J. C. (1976) *Biochemistry* **15,** 5776–5783
22. Gordon, R. L., Humphries, P., and McDonnell, D. J. (1978) *Mol. Gen. Genet.* **162,** 329–339
23. Siebenlist, U. (1979) *Nucleic Acids Res.* **6,** 1895–1907
24. McConnell, D. J. (1979) *Nucleic Acids Res.* **6,** 525–544
25. Dunn, J. J., and Studier, F. W. (1981) *J. Mol. Biol.* **148,** 303–330
26. Johnston, D. E., and McClure, W. R. (1976) in *RNA Polymerase* (Losick, R., and Chamberlin, M. eds) pp. 413–428, Cold Spring Harbor Laboratory, New York
27. Cech, C. L., Lichy, J., and McClure, W. R. (1980) *J. Biol. Chem.* **255,** 1763–1766
28. McGhee, J. D., and von Hippel, P. H. (1974) *J. Mol. Biol.* **86,** 469–489
29. Hawley, D. K., and McClure, W. R. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 6381–6385
30. Stefano, J. E., and Gralla, J. D. (1982) *J. Biol. Chem.* **257,** 13924–13929
31. Rosenberg, S., Kadesch, T. R., and Chamberlin, M. J. (1982) *J. Mol. Biol.* **155,** 31–51
32. Chamberlin, M. J., Rosenberg, S., and Kadesch, T. (1982) in *Promoters* (Rodriquez, R. L., and Chamberlin, M. J., eds) pp. 34–53, Praeger, New York
33. Shih, M., and Gussin, G. N. (1982) in *Promoters* (Rodgriquez, R. L., and Chamberlin, M. J., eds) pp. 96–110, Praeger, New York
34. McClure, W. R., Hawley, D. K., and Malan, T. P. (1982) in *Promoters* (Rodriquez, R. L., and Chamberlin, M. J., eds) pp. 111–120, Praeger, New York
35. McConnell, D. J. (1979) *Nucleic Acids Res.* **6,** 525–544
36. Siebenlist, U., Simpson, R. B., and Gilbert, W. (1980) *Cell* **20,** 269–281
37. de Haseth, P. L., Lohman, T. M., Burgess, R. R., and Record, M. T., Jr. (1978) *Biochemistry* **17,** 1612–1622
38. Hawley, D. K., and McClure, W. R. (1982) *J. Mol. Biol.* **157,** 493–525
39. Pfeffer, S. R., Stahl, S. J., and Chamberlin, M. J. (1977) *J. Biol. Chem.* **252,** 5403–5407
40. Strauss, H. S., Burgess, R. R., and Record, M. T., Jr. (1980) *Biochemistry* **19,** 3504–3515
41. Kadesch, T. R., Rosenberg, S., and Chamberlin, M. J. (1982) *J. Mol. Biol.* **155,** 1–29
42. Carpousis, A. J., and Gralla, J. D. (1980) *Biochemistry* **19,** 3245–3253
43. Munson, L. M., and Reznikoff, W. S. (1981) *Biochemistry* **20,** 2081–2085
44. McClure, W. R., and Cech, C. L. (1978) *J. Biol. Chem.* **253,** 8949–8956
45. Nierman, W. C., and Chamberlin, M. J. (1980) *J. Biol. Chem.* **255,** 1819–1823