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Bacteriophage T7 E Promoter: Identification and Measurement of Kinetics of Association with *Escherichia coli* RNA Polymerase†

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ABSTRACT: The initiation point for transcription from the *Escherichia coli* RNA polymerase E promoter on bacteriophage T7 has been determined to be at 36 835 base pairs (92.22 T7 units) from the left end of T7. The location was determined by RNA fingerprinting of a runoff transcription product. Kinetics of association for the E and the T7 A3 promoters were measured by using the abortive initiation assay for approach to steady-state turnover. The kinetic association constant, k_a ($=K_B k_2$), for E was found to be over 10-fold slower than k_a for A3. For the E promoter, $k_a = 1.2 \times 10^6$ M^{-1} s^{-1}. For A3, we report $k_a \geq 4 \times 10^7$ M^{-1} s^{-1}. This difference is due mostly to a 10-fold difference in the initial equilibrium constant, K_B, for formation of the initial polymerase–promoter complex. The rate of isomerization, k_2, of the initial complex to the open polymerase–promoter complex for the E promoter was only 2-fold slower than k_2 for the A3 promoter. Various numerical methods for calculation of the kinetic parameters are discussed and compared. We argue that a nonlinear analysis provides the most reliable means of data analysis.

Regulation of transcription is essential for the control of gene expression. Transcriptional regulation can occur by the presence or absence of effector molecules or simply by the differential affinities of promoters for RNA polymerase (RNAP).[1] Bacteriophage T7 is a standard example of the latter. During infection of *Escherichia coli*, the early region of T7 is transcribed by RNAP from the three tandem A promoters (A1, A2, and A3) at the left end of T7 (see Figure

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[1] Abbreviations: RNAP, RNA polymerase; poly[d(A-T)], poly[d(A-T)·d(A-T)]; poly[d(I-C)], poly[d(I-C)·d(I-C)]; DTT, dithiothreitol; bp, base pair(s); RNase, ribonuclease; TE, 10 mM Tris, pH 8 at 25 °C, and 1 mM EDTA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PEI, poly(ethylenimine); TLC, thin-layer chromatography.

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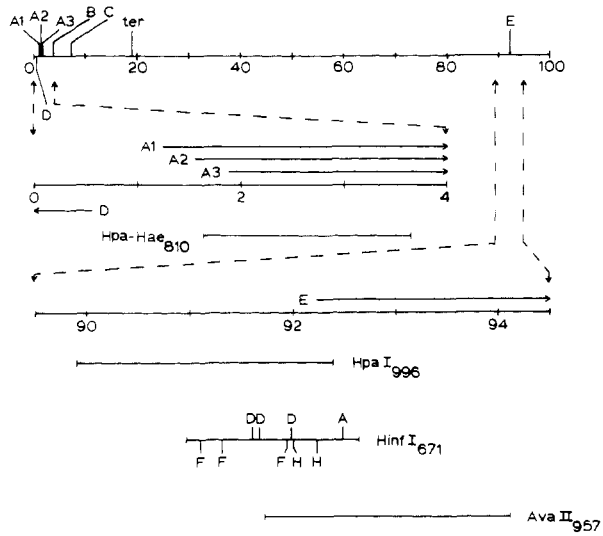

FIGURE 1: Transcription map of T7 for *E. coli* RNA polymerase. The A promoters are active in vivo early in infection. B, C, D, and E promoters are only known to transcribe in vitro. The genome is normalized to 100 units (1 T7 unit = 399.36 bp). Restriction fragments used in this work are also shown. Fragment lengths in base pairs (shown as subscripts) were determined from computer-assisted analysis of the DNA sequence (Dunn & Studier, 1983). Subsequent verification of fragment identity was determined by restriction patterns of isolated fragments. Restriction endonuclease cleavage sites on the *Hinf*I$_{671}$ fragment are *Alu*I (A), *Dde*I (D), *Fnu*4HI (F), and *Hae*III (H).

1). The gene products from this transcription unit include both a protein which inactivates *E. coli* RNAP and a T7-specific RNA polymerase which transcribes the middle and late regions of T7. Transcripts from the three A promoters are the only *E. coli* RNAP transcripts which have been detected in vivo (Dunn & Studier, 1973; Kramer et al., 1974; Hercules et al., 1976). The "major" A promoters also predominate in vitro at low RNAP concentrations. At higher but less than saturating concentrations of RNAP, the "minor" B, C. D, and E promoters are transcribed efficiently (Stahl & Chamberlin, 1977; Wiggs et al., 1979; Cech & McClure, 1980). Initiation of transcription can be described by the following minimal sequence of events: (1) initial binding of RNAP to the DNA promoter, forming the initial RNAP–promoter complex (RP_i); (2) isomerization of RP_i into an open complex (RP_o) in which disruption of the double-stranded DNA helix has occurred near the initiation site (Siebenlist, 1979a); (3) binding of nucleoside triphosphates to RP_o and formation of the nascent RNA chain. This may be written schematically (McClure, 1980; Stefano & Gralla, 1980) 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 R represents RNAP, P is the promoter, k_2 is the isomerization rate for the conversion of initial to open complex, and $K_B = k_1/k_{-1}$ is the equilibrium binding constant for the initial complex, RP_i. We previously reported that the major T7 A1 and A3 promoters differ from the minor C and D promoters in that the initial binding constant, K_B, for the A promoters is at least 10-fold larger than that for the minor promoters (Dayton et al., 1984).

Transcription initiation sites for the three major A promoters and two of the minor promoters (C and D) have been sequenced directly (Pribnow, 1975; McConnell, 1979; Rosenberg & Court, 1979; Siebenlist, 1979b; Zaichikov & Pletnev, 1980). However, the location of the E promoter has only been tentatively established. Electron microscopy and transcript length studies (Delius et al., 1973; Stahl & Chamberlin, 1977; Koller et al., 1978; Kadesch et al., 1980) indicate the E promoter is located between 91 and 93.2 T7 units. After sequencing the entire T7 genome (39 936 bp), Dunn & Studier (1983) proposed that the E promoter corresponds to a sequence at 92.47 T7 units.

In this paper, we report the exact location of the E promoter and its kinetic parameters. In addition to measuring the kinetic parameters for the E promoter, we also remeasured the A3 promoter kinetics for two reasons. We wished to obtain sufficient data to reduce the relative error in earlier measurements of A promoters, and we chose a fragment in which the left end was not as close to the −35 region of the promoter. We also discuss the various graphical and numerical methods for calculating the kinetic parameters, k_2 and K_B, from experimental data.

Materials and Methods

Polymerase and DNA. RNA polymerase (RNAP) was isolated from *E. coli* (Grain Processing) by the methods of Burgess & 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 was reported here, unless otherwise indicated. Bacteriophage T7 was grown in *E. coli* and the DNA isolated by standard procedures (Cech & McClure, 1980). Poly[d(A-T)] and poly[d(I-C)] were purchased from P-L Biochemicals. 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 digestion of *N,N'*-bis(acrylylcystaminylacrylamide) gels with 2-mercaptoethanol (Hansen, 1976). Contaminating acrylamide was removed by loading DNA solutions onto DEAE-A25 Sephadex columns and rinsing with 0.1 M KCl. DNA was dialyzed into TE (10 mM Tris, pH 8 at 25 °C, and 1 mM EDTA). Promoter-containing fragments were identified by using DELILA computer programs (Schneider et al., 1982; Stormo et al., 1982) to search T7 sequence data (Dunn & Studier, 1983) for restriction nuclease cleavage sites. Fragments referred to in this paper are shown in Figure 1.

Abortive Initiation Assays. The principle and properties of the abortive initiation assay have been reported (Johnston & McClure, 1976; Cech et al., 1980). Briefly, abortive initiation is the reiterative production of a short oligonucleotide, usually a dinucleoside tetraphosphate, when only two or three nucleoside triphosphates are present in the transcription reaction. Nucleoside monophosphates and dinucleoside monophosphates also can function as the initiating nucleotides in abortive initiation reactions. The primary abortive initiation products used in these kinetic studies were pApC and CpApU for the 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). Other buffers used were buffer KCl-50 (same as buffer KCl-80 except at 50 mM KCl) etc.

To measure association constants, the appropriate combination of starting nucleoside monophosphate or dinucleoside monophosphate (1 mM) and the elongating nucleoside triphosphate (0.04 mM) with the corresponding α-^{32}P-labeled nucleotide added at 2000 cpm/pmol of elongating nucleotide was preincubated for 10 min at 37 °C with 1 nM DNA template. Polymerase, ranging in concentration from 25 to

150 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. Nucleoside triphosphates were then added at time zero and aliquots of the reaction measured for product formation as a function of time.

To obtain dissociation rates, the methods described by Cech & McClure (1980) were followed. Dissociation was measured by preincubating 80 nM RNAP with 1–3 nM template for 10 min at 37 °C prior to addition of heparin (final concentration 500 μg/mL) or competing synthetic copolymer DNA [0.05 or 0.2 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 10000 cpm/pmol of UTP. RNAP, 8–16 pmol, was preincubated for 10 min with 0.15 pmol of promoter-containing DNA fragment. Nucleotides were then added to the reaction, giving a final volume of 50 μL. After 4 min, heparin was added to 40 μg/mL to prevent further reinitiation. The reaction was allowed to proceed for an additional 25 min to assure that all transcription was complete. For transcriptional salt dependence studies of the E promoter, [KCl] was varied from 50 to 200 mM.

Fingerprint Analysis. Transcripts for fingerprinting were labeled with all four [α-^{32}P]NTP's at 20 μM in buffer KCl-80. These transcripts were isolated from 5% polyacrylamide–7 M urea gels by crush and soak methods similar to those described by Maxam & Gilbert (1980), except the crush and soak buffer was 10 mM Tris, pH 8, 0.5 M NaCl, 5 mM EDTA, and 1% phenol. The eluted RNA was ethanol precipitated and washed twice with 70% ethanol. Digestion with RNase T1 was performed in 5 μL of TE with 100 μg/mL RNase T1 (Gruissem et al., 1982; Zaug & Cech, 1982). Two-dimensional analysis, fingerprinting, was performed by electrophoresis on cellulose acetate paper strips in the first direction and poly(ethylenimine) (PEI)–cellulose thin-layer chromatography in the second dimension (Barrel, 1971; Gruissem et al., 1982; Zaug & Cech, 1982). The fingerprints were visualized by autoradiography.

T1 oligonucleotides from the above fingerprints were isolated from the PEI–cellulose with 70 μL of 2 M triethylamine bicarbonate and washed 3 times with 50 μL of water (Gruissem et al., 1982; Zaug & Cech, 1982). Digestion of the T1 oligonucleotides by pancreatic RNase was performed in 15 μL of TE containing 0.4 μg/mL pancreatic RNase.

Identification of the pancreatic RNase oligonucleotides was performed on PEI plates as described by Volckaert & Fiers (1977). The plates were developed with 22% formic acid in the first direction and with 1:1 M formic acid adjusted with pyridine to pH 4.3 in the second dimension. Secondary two-dimensional analyses were also visualized by autoradiography. Some T1 oligonucleotides were also digested with P1 RNase (400 μg/mL in 5 μL of TE) for terminal triphosphate analysis. These digestion analyses were performed on one-dimensional PEI plates and developed as described by Zaug & Cech (1982), except with 1.6 M LiCl instead of 1.0 M LiCl.

Mathematical Calculations. Linear least-squares programs were similar to those outlined by Bevington (1969). Nonlinear least-squares programs utilized the Marquardt algorithm (Bevington, 1969). Confidence intervals are normally reported as ±1 standard deviation (a 67% confidence interval). However, the confidence intervals are often asymmetric for nonlinear equations (Johnson, 1983). A statistical F test was used to determine the values of the parameters at a 67% confidence level (Bevington, 1969).

The observed association constants, k_{obsd}, were determined by the nonlinear regression program using the equation of McClure (1980):

$$[\text{prod}] = k_3 t - (k_3/k_{obsd})(1 - e^{-k_{obsd}t}) \quad (1)$$

where [prod] is the concentration of abortive product in the reaction at time t. Assuming that $k_{-1} \gg k_2$ (preequilibrium condition), that $k_{-2} \ll k_2$ [see Cech & McClure (1980) and Dayton et al. (1984) for supporting evidence], that [RNAP] $\gg$ [promoter], and that $[RP_i]$ is at steady state, $d[RP_i]/dt = 0$, then $1/k_{obsd}$ reduces to

$$1/k_{obsd} = \tau = 1/k_2 + 1/k_2 K_B[\text{RNAP}] \quad (2)$$

(McClure, 1980). A plot of τ vs. 1/[RNAP] is referred to by McClure (1980) as a tau plot. The other linear and nonlinear equations used here can be easily derived from eq 2. They are as follows:

$$k_{obsd} = k_2 - (1/K_B)(k_{obsd}/[\text{RNAP}]) \quad (3)$$

$$[\text{RNAP}]/k_{obsd} = 1/k_2 K_B + (1/k_2)[\text{RNAP}] \quad (4)$$

$$k_2 = k_{obsd} + (1/K_B)(k_{obsd}/[\text{RNAP}]) \quad (5)$$

$$k_{obsd} = k_2[\text{RNAP}]/([\text{RNAP}] + 1/K_B) \quad (6)$$

$$k_{obsd} = k_2[\text{RNAP}]/([\text{RNAP}] + k_2/k_a) \quad (7)$$

Equations 2, 3, 4, 5, and 6 are referred to here as the double-reciprocal, Eadie–Hofstee, Woolf, Cornish-Bowden, and nonlinear equations, respectively. These equations are analogous to the Michaelis–Menten equations cited by Dowd & Riggs (1965), Cornish-Bowden & Eisenthal (1974), Currie (1982), and Lam (1981). The value of k_a for each promoter was calculated by using eq 7. Estimates of k_{-2} were calculated by using a nonlinear regression program for a pseudo-first-order dissociation reaction.

With a pseudo-random-number generator, data sets were generated with a Gaussian distribution about the theoretical data points. The theoretical curve fit eq 6 with $K_B = 2.8 \times 10^7$ M^{-1} and $k_2 = 0.043$ s^{-1}. Eight RNAP concentrations corresponding to those in Figure 6B were used. Two types of randomly generated data were used to compare analysis methods derived from eq 2–6. Each type of generated data consisted of 50 sets of 8 data points. The first type, "10% error data", was generated with a random relative error in k_{obsd} of 10% for all points. The second type, "33%/10% error data", was generated with a random relative error in k_{obsd} of 33% for the lowest RNAP concentration and 10% for all the other RNAP concentrations.

Results

Location. We identified potential E promoters in the region near the reported E transcript origin (Delius et al., 1973; Stahl & Chamberlin, 1977; Koller et al., 1978; Kadesch et al., 1980). We used the following two computer sequence search methods: (a) the "pattern learning" program in the Delila system, which incorporates the perceptron algorithm (Stormo et al., 1982; Doug Kemme, unpublished results); (b) a search for the consensus sequence TTGACA...16–18 bp...TATAAT where

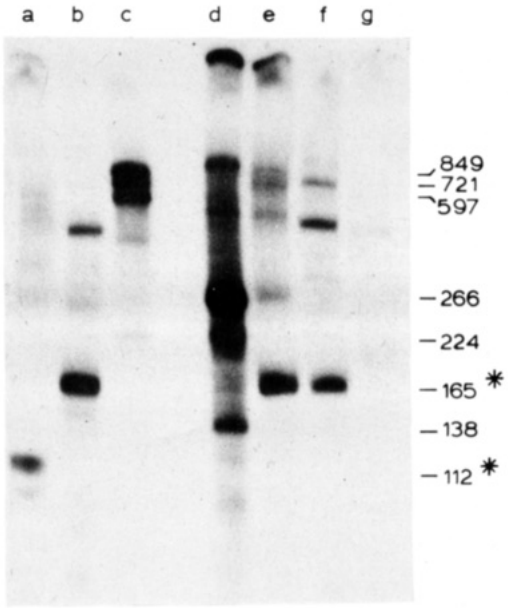

FIGURE 2: Runoff transcription from the *Hin*fI$_{671}$ fragment and fragments derived from *Hin*fI$_{671}$. All reactions were run in buffer KCl-80 with 3 nM DNA template and 150 nM active RNAP. The following restriction fragments were used as templates: (a) *Hin*fI$_{671}$ digested with *Alu*I; (b) *Hin*fI$_{671}$ digested with *Dde*I; (c) 1599 bp C5 *Hae*III left end; (d) 763 bp *Hin*fI left end; (e) *Hin*fI$_{671}$; (f) *Hin*fI$_{671}$ digested with *Fnu*4HI; (g) *Hin*fI$_{671}$ digested with *Hae*III. Fragments in lanes c and d each contain the A1, A2, A3, and D promoters. Transcripts from these fragments were used as RNA length standards. Standards are the 849-nucleotide A1 transcript (lane c), 721-nucleotide A2 transcript (lane c), 597-nucleotide A3 transcript (lane c), 224-nucleotide D transcript (lanes c and d), 266-nucleotide A1 transcript (lane d), and 138-nucleotide A2 transcript (lane d). Transcript lengths designated with an asterisk were calculated relative to the standards.

up to four deviations (mismatches) from the consensus sequence were allowed. Both methods used the Delila data base and programs (Schneider et al., 1982). The most likely sequence contained the highly conserved T (italicized in the consensus sequence) at 36 827 bp from the left end of the T7 genome (Figure 1). We will make use of two nomenclatures to designate positions on the T7 genome: bp from the left end (1–39 936) and T7 units (0–100, where 0 is the left end and 100 the right end of the T7 genetic map) (Dunn & Studier, 1983).

The region containing the potential E promoter was isolated on three different fragments, a 671 bp *Hin*fI fragment, a 996 bp *Hpa*I fragment, and a 957 bp *Ava*II fragment. Collectively, these three fragments span from 89.9 to 94.1 T7 units. The major runoff transcript from each DNA fragment was approximately 160, 67, and 755 bases, respectively (data not shown). These lengths are consistent with a promoter at 36 827 (Figure 1).

The *Hin*fI$_{671}$ fragment was digested with various restriction enzymes (Figure 1). DNA from each digest was transcribed (Figure 2). Each transcription product is consistent with a promoter starting at 36 835 ± 10 bp. A 160-base runoff transcript from the *Dde*I digestion–transcription reaction and a 160-base runoff transcript from the *Fnu*4HI digestion–transcription reaction show that the major transcript does not arise from an internal promoter–terminator pair within the *Hin*fI$_{671}$ fragment nor is it a runoff transcript in the leftward direction. The shorter 102-base runoff transcript from the *Alu*I digestion–transcription reaction is consistent with a rightward runoff transcript originating at 36 835 ± 10 bp. No major transcript was observed from the *Hae*III digestion–transcription reaction, as expected, since *Hae*III cuts at −2 of

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          -35                -10        +1
          TTGACA             TATAAT
AAAAAGAGTA TTGACT TAAAGTCTAACCTATAG GATACT TACAGCC ATCGAGAGGG   A1
AAAACAGGTA TTGACA ACATGAAGTAACATGCAGTAAGAT ACAAATC GCTAGGTAAC   A2
AACAAAACGG TTGACA ACATGAAGTAAACACGG TACGAT GTACCAC ATGAAACGAC   A3
GATAAGCAAC TTGACG CAATGTTAATGGGCTGA TAGTCT TATCTT  ACAGGTCATC   C
CTTACGGATG ATGATA TTTACACATTACAGTGA TATACT CAAGGCC ACTACAGATA   E
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FIGURE 3: T7 promoter sequences taken from Dunn & Studier (1983). The most frequently observed bases (consensus sequence) (Rosenberg & Court, 1979; Siebenlist et al., 1980) are shown for comparison. Position +1 corresponds to the transcription initiation sites.

Table I: Oligonucleotide Assignments for the Fingerprint Shown in Figure 4

T1 RNase oligonucleotide	pancreatic RNase oligonucleotide	deduced[a] sequence
1	AC$_n$,AG,C,U,NTP[b]	pppACUACAGp
2	AG,AU	AUAGp
3	G,U	UGp
4	G	Gp
5	AU,C,G,U$_n$	UCUUUAUGp
6	AU,G	AUGp
7	AU,C,G,U$_2$	UCAUUGp
8	AC,AU,C,G,U$_n$	UCUAUACGp
9	AG	AGp
10	AAAU,AC,AU,C,G,U	AAAUCUGp
11	AC,C$_n$,G,U$_n$	CUCCUACGp
12	AAAG	AAAGp

[a] Deduced from the T7 DNA sequence: (CC)A$^{36\,835}$CTACAGATAGTGGTCTTTATGGATGTCATTGTC-TATACGAGATGCTCCTACGTGAAATCTGAAAG$^{36\,900}$. [b] The presence of 5′-triphosphates was verified by P1 digestion analysis of the 5′-T1 and 5′-T1-pancreatic oligonucleotides. The 5′-triphosphates were ATP and CTP ([ATP] > [CTP]).

the E promoter. Upon longer exposure, we were able to detect a very faint band corresponding to an RNA of about 60 bases in all lanes except the *Alu*I lane. This is consistent with a transcript initiating from the 36 927 promoter proposed by Dunn & Studier (1983).

Stahl & Chamberlin (1977) used [γ-^{32}P]GTP and [γ-^{32}P]ATP to determine that the E promoter transcript is initiated with ATP. We investigated the 5′ end of the transcript from the *Hin*fI$_{671}$ fragment using the abortive initiation assay (Johnston & McClure, 1976; Cech et al., 1980). Using different combinations of reactants, we were able to detect three abortive initiation products corresponding to two or three unique start sites (Figure 3). The pApC and CpApC abortive initiation products correspond to the same start site, at 36 835. We were not able to determine whether a third product was pppCpApC or pppCpCpApC or a combination of both. This product will be referred to as the pppCpApC–pppCpCpApC product. The dinucleotide pApC was the major product. The turnover rate for pApC was 5-fold greater than the CpApC turnover rate and 3–5-fold greater than the pppCpApC–pppCpCpApC product turnover rate. The A1 promoter (on a 536 bp *Hpa*II fragment) produces a pApU product at a rate twice that for its CpApU product. Conversely, the A3 promoter exhibits a CpApU product turnover rate 8-fold faster than the rate for its corresponding pApU product.

To confirm the location of the E promoter, fingerprint analysis was performed on the 66-base RNA transcript from the *Hpa*I$_{996}$ fragment. The transcript was body labeled with all four α-^{32}P-nucleotides. All major T1 oligonucleotides were isolated from fingerprint plates (Figure 4). Aliquots from all major T1 oligonucleotides were digested with pancreatic RNase and analyzed by two-dimensional PEI TLC as described for secondary digestions. The oligonucleotide sequences were deduced from the pancreatic RNase digestion products

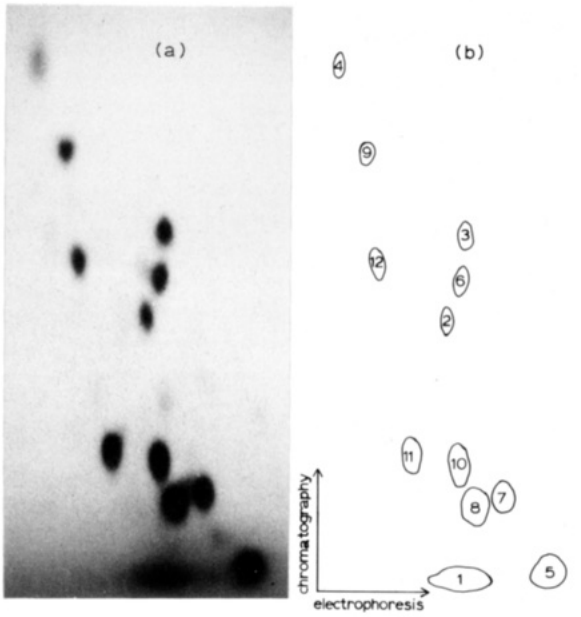

FIGURE 4: Fingerprint analysis of the ribonuclease T1 digestion products of the 66-nucleotide E promoter runoff transcript. (a) Autoradiogram of PEI chromatogram. (b) Diagrammatic representation of the fingerprint in (a) identifying by number the oligonucleotides in Table I. Electrophoresis on cellulose acetate and poly(ethylenimine) thin-layer chromatography procedures are described under Materials and Methods.

and the known DNA sequence (Table I). Aliquots of T1 oligonucleotides 1, 5, 7, 8, 10, and 11 were also digested with P1 RNase and separated by one-dimensional PEI TLC to identify the nucleoside triphosphate base of the 5′-oligonucleotide. ATP and some CTP were detected in the P1 digests of fragment 1. Thus, both ATP and CTP can be used by RNAP to initiate transcription at the E promoter in vitro. The ATP-initiated transcript begins from 36 835. The CTP-initiated transcript most likely begins from 36 834. However, CTP initiation at 36 833 or 36 836 cannot be ruled out.

Two other transcript bands on the 5% polyacrylamide–7 M urea gel, both running somewhat faster than the main band, were isolated. Both had fingerprints indistinguishable from that of the main band. Since the potential 3′-terminal U or UpU (*Hpa*I end) was not detected, these bands could have differed by one or two nucleotides. However, electrophoretic mobilities corresponded to differences of four and six nucleotides from the main transcript. Most likely, the RNA in these bands contained secondary structure features despite efforts to prevent renaturation.

Salt Dependence. It is known that increasing ionic strength has a greater inhibitory effect on transcriptional initiation than on elongation (Chamberlin, 1976). The abortive initiation turnover rate profile of the pApC product as a function of [KCl] was studied for the E promoter (Figure 5). The maximum abortive turnover rate occurred with no KCl present (10 mM $MgCl_2$ was present in the reaction mixture). Nearly identical results were obtained for the pApC product of the C promoter (data not shown). These results resemble those of McClure et al. (1978) for pppApU production from λ b2 DNA.

The effect of ionic strength on the transcriptional utilization of the E promoter by RNAP was also investigated. It had been reported earlier that the E promoter was utilized by RNAP for transcription only below 100 mM KCl (Stahl & Chamberlin, 1977; Wiggs et al., 1979). However, we observed an equivalent production of runoff transcript from the E promoter on the *Hin*fI$_{671}$ fragment at 200 and 50 mM KCl (Figure 5).

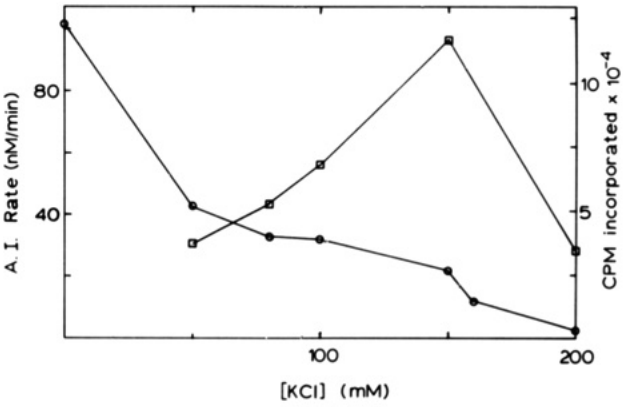

FIGURE 5: Salt dependence of T7 E promoter activity. Buffers for the abortive initiation reactions (○) were the same as buffer KCl-80 except the KCl concentration was varied from 0 to 200 mM. The *Hin*fI$_{671}$ fragment at 1 nM was preincubated with 80 nM RNAP for either 10 or 20 min before addition of AMP and CTP, as described under Materials and Methods. Aliquots of these reactions were removed and analyzed after 15 and 30 min. Buffers for the runoff transcription (□) were the same as buffer KCl-80 except the [KCl] was varied from 50 to 200 mM. Runoff transcription reactions were as described under Materials and Methods.

Table II: Comparison of Numerical Methods for Calculating Kinetic Parameters

	E promoter		A3 promoter	
method[a]	k_2 (s^{-1})	K_B (M^{-1})	k_2 (s^{-1})	K_B (M^{-1})
double reciprocal	0.122	6.1×10^6	0.099	1.2×10^8
Eadie–Hofstee	0.020	-1.7×10^9	0.058	-1.7×10^8
Woolf	0.039	3.7×10^7	0.093	1.6×10^8
Cornish-Bowden	0.042	2.2×10^7	0.15	3.7×10^8
nonlinear	0.043	2.8×10^7	0.087	4.7×10^8

[a] Values shown were calculated from E promoter experimental data without use of weighting factors. For the E promoter data, double-reciprocal analysis with weighting factors gives $k_2 = 0.034\ s^{-1}$ and $K_B = 6.6 \times 10^7\ M^{-1}$.

The [KCl] for maximum utilization occurred near 150 mM.

Kinetics. The kinetic parameters were determined for both the minor E and the major A3 promoters. All measurements of association kinetics for E were performed with the *Hin*fI$_{671}$ fragment (Figure 1). Dissociation kinetic measurements were performed with the *Hin*fI$_{671}$ or *Ava*II$_{957}$ fragments. The A3 promoter was studied by using an 810 bp, *Hpa*II to *Hae*III, fragment (*Hpa–Hae*$_{810}$; Figure 1). The kinetic parameters k_2 and K_B (where $k_2K_B = k_a$) were calculated as described under Materials and Methods.

The kinetic constants for the E and A3 promoters calculated by five different methods are shown in Table II. The nonlinear method has weighting factors equal to a constant over the entire range (Lam, 1981). For the double-reciprocal method, the weighting factor is $(k_{obsd})^4$. Use of proper weighting functions and data with relatively little scatter should, in theory, largely render these methods equally reliable. However, weighting factors are commonly not used at all in double-reciprocal analysis (Dowd & Riggs, 1965). In practice, we found the double-reciprocal linear regression analysis without weighting factors was very sensitive to outlying points. With weighting factors, the sensitivity to outlying points is biased in favor of higher values of k_{obsd}. The double-reciprocal analysis (with and without weighting factors) for the E promoter k_{obsd} data, for example, is plotted in Figure 6A along with a line calculated from parameters generated by a nonlinear analysis of the same data.

Dayton et al. (1984) reported a 10–20% estimated error in the values of τ. With the randomly generated 10% error data,

Table III: Numerical Methods Applied to Randomly Generated Data[a]

method	k_2 (s^{-1})	K_B (M^{-1})
theoretical value	0.043	2.8×10^7
double reciprocal	0.0278 ± 0.115	$(3.24 \pm 2.53) \times 10^7$
weighted[b] double reciprocal	0.0402 ± 0.0073	$(4.16 \pm 2.04) \times 10^7$
Eadie–Hofstee	0.0310 ± 0.0076	$(8.26 \pm 8.27) \times 10^7$
Woolf	0.0409 ± 0.0125	$(2.97 \pm 1.58) \times 10^7$
Cornish-Bowden	0.0393 ± 0.0081	$(3.32 \pm 1.31) \times 10^7$
nonlinear	0.0432 ± 0.0088	$(3.28 \pm 1.55) \times 10^7$

[a] Values of k_2 and K_B represent the mean calculated from 50 33%/10% data sets. Standard deviation from the mean is also shown. The distributions were not Gaussian. [b] The weighting factor was $(k_{obsd})^4$ as discussed in the text.

only the Eadie–Hofstee method did not accurately approximate the theoretical values (data not shown). For the E promoter, the relative error in k_{obsd} for the lowest RNAP concentration was 33%. Using the randomly generated 33%/10% error data (10% for all but the lowest [RNAP] data point), the double-reciprocal method with and without weighting factors, and the Eadie–Hofstee methods less accurately approximated the theoretical values for K_B and k_2 (Table III). These methods have also been shown to be less reliable in the analysis of Michaelis–Menten enzyme kinetics (Dowd & Riggs, 1965; Lam, 1981; Currie, 1982). Woolf, Cornish-Bowden, and nonlinear methods more accurately approximated the parameters. For these methods, the standard deviation from the mean for the K_B and k_2 values calculated from the randomly generated data sets was less than the 67% confidence limits calculated from the actual data (Table IV). We estimate that the relative error in measurements of k_{obsd} is about 20% with somewhat larger error at lower RNAP concentrations. Since the Woolf and Cornish-Bowden methods do not lend themselves to determination of confidence intervals, we have chosen to use kinetic parameters derived by use of the nonlinear equation (eq 6).

Values of k_a, k_2, and K_B for experimental data along with 67% confidence levels are listed in Table IV. For the E promoter, $k_2 = 0.043\ s^{-1}$, $K_B = 2.8 \times 10^7\ M^{-1}$, and $k_a = 1.2 \times 10^6\ M^{-1}\ s^{-1}$. For the A3 promoter, $k_2 = 0.087\ s^{-1}$, $K_B \geq 4.7 \times 10^8\ M^{-1}$, and $k_a \geq 4 \times 10^7\ M^{-1}\ s^{-1}$. A nonlinear fit of the E promoter data along with 67% confidence levels is shown in Figure 6B. With the use of k_2 and K_B determined from a weighted double-reciprocal analysis, the predicted nonlinear curve is within the 67% confidence intervals shown in Figure 6B. The line, however, is above rather than through all but the two highest points. The k_2 value determined by unweighted double-reciprocal analysis is outside the 67% confidence intervals determined by the nonlinear analysis. Figure 7 shows

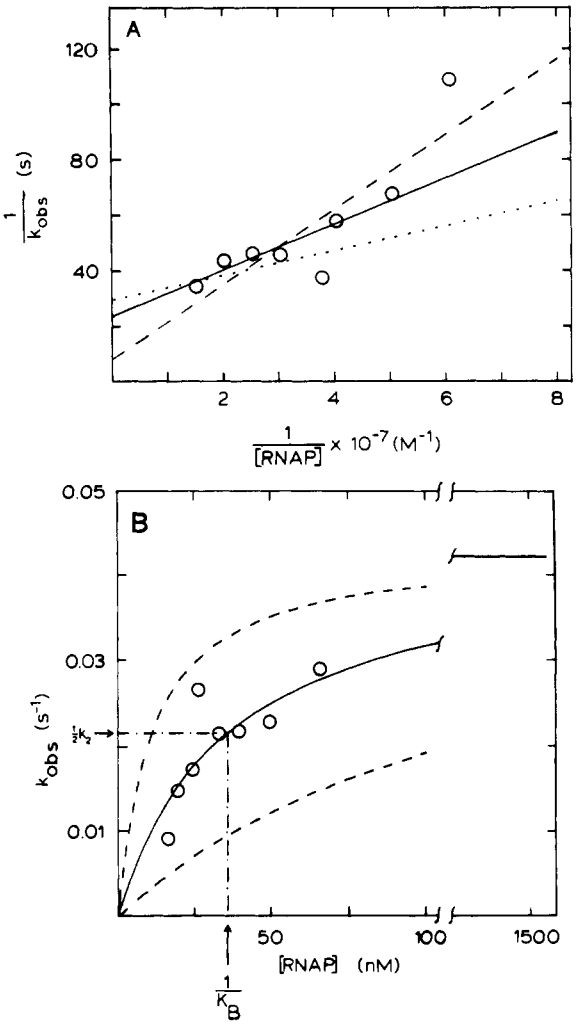

FIGURE 6: Nonlinear and tau plots of E promoter kinetics of association. (A) Tau plot (McClure, 1980) of k_{obsd} as a function of active RNAP concentration: (—) curve generated by parameters calculated according to eq 6 and the nonlinear regression program; (– –) curve generated by parameters calculated by using eq 2 and a linear regression program without weighting factors; (···) curve generated by parameters calculated by using eq 2 and a linear regression program with weighting factors. (B) Same data showing the curve fitted (—) by nonlinear regression analysis. The dashed curves (– –) represent $K_B \pm$ a 67% confidence level and $k_2 = 0.043\ s^{-1}$. k_2 = maximum k_{obsd}, $K_B = 1/[RNAP]$ at 0.5 k_2. All reactions contained the *Hin*fI$_{671}$ fragment at 1 nM in buffer KCl-80.

the same nonlinear analysis applied to the A3 data. McClure (1980) and Dayton et al. (1984) have indicated that because the relative error is large for the nearly flat slope of A promoter

Table IV: Kinetic Parameters of the E and A3 Promoters

	E promoter			A3 promoter		
		67% confidence interval			67% confidence interval	
constant	value	lower limit	upper limit	value	lower limit	upper limit
k_2 (s^{-1})	0.043	0.031	0.071	0.087	0.064	0.155
K_B (M^{-1})	2.8×10^7	1.2×10^7	8.3×10^7	4.8×10^8	0.4×10^8	∞[a]
k_a ($M^{-1}\ s^{-1}$)	1.2×10^6	0.8×10^6	2.0×10^6	4.1×10^7	0.6×10^7	∞[a]
$t_{1/2}$ for k_d (min)[b]	13	9.6	22	ND[c]		
k_{-2} (s^{-1})[b]	8.6×10^{-4}	5.2×10^{-4}	12×10^{-4}	ND		
$K_{eq} = k_a/k_{-2}$ (predicted, M^{-1})[d]	1.4×10^9			ND		
$t_{1/2(heparin)}$ (min)[e]	0.85	0.69	1.1	23	20	27
$k_{-2,heparin}$ (s^{-1})	0.014	0.011	0.017	5.0×10^{-4}	4.3×10^{-4}	5.8×10^{-4}
$K_{eq,heparin}$ (predicted, M^{-1})	8.8×10^7			8.2×10^{10}		

[a] The upper limit at a 67% confidence level had not been reached at 10^{38}. [b] Dissociation experiments performed with poly[d(A-T)] as a sequestering agent. k_{-2} confidence intervals were determined by normal standard deviation calculations from triplicate or quadruplicate k_{-2} experiments. [c] ND, not determined. [d] Predicted equilibrium constants using k_{-2} from footnote *b*. [e] Heparin subscripts denote use of heparin as a sequestering agent for k_{-2} determination.

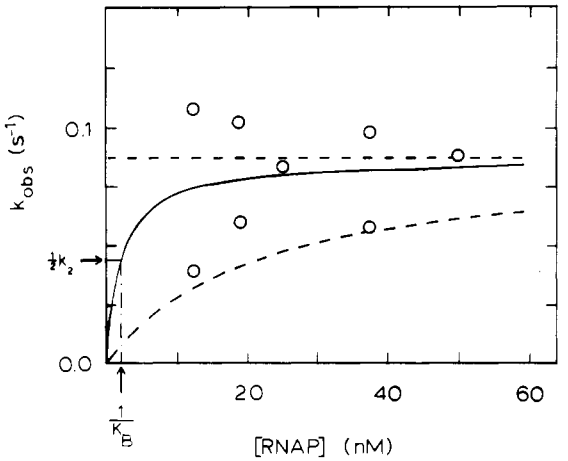

FIGURE 7: Nonlinear plot of k_{obsd} as a function of active [RNAP] for the A3 promoter. (—) Curve generated by parameters calculated according to eq 6 and the nonlinear regression program (Table IV). The dashed curves (– –) represent $K_B \pm$ a 67% confidence level and $k_2 = 0.087\ s^{-1}$. (The upper dashed curve represents $K_B = 10^{38}\ M^{-1}$.) All reactions contained the *Hpa–Hae*$_{810}$ fragment at 1 nM.

tau plots, K_B for these promoters represents only a lower limit. When we used the statistical F test to determine confidence levels for the A3 promoter K_B parameter, a confidence of only 51% had been reached at $K_B = 10^{38}$ (upper limit of the computer).

Given the condition that $k_{-1} \gg k_2, k_{-2}$ equals the dissociation rate, k_{off}, observed when RP_o is challenged with a sequestering agent for free polymerase (McClure, 1980; see Materials and Methods). The dissociation rate for the E promoter was determined by using two different sequestering agents, heparin and poly[d(A-T)]. We determined the $t_{1/2}$ of dissociation to be approximately 0.9 min using heparin and 13 min using poly[d(A-T)]. The $t_{1/2}$ of dissociation for A3 using heparin as the sequestering agent was determined to be 23 min.

Discussion

We have located the T7 E promoter by analysis of transcriptional products after an initial computer-assisted search for potential promoter sites. The region containing the best promoter-like sequence was isolated on overlapping restriction enzyme fragments spanning from 89.9 to 94.8 T7 units (35 904–37 589 bp) on the T7 genome. Dunn & Studier (1983) proposed that the E promoter was located at 36 927. The reasons for this assignment were as follows: (a) electron microscopy studies have placed the position near 91–93 T7 units, or between 36 340 and 37 140 bp (Delius et al., 1973; Stahl & Chamberlin, 1977; Koller et al., 1978; Kadesch et al., 1980); (b) experiments reported by Allet et al. (1974) showed that binding of *E. coli* RNA polymerase to T7 DNA blocked certain *Hin*dII + *Hpa*I cleavages. Dunn & Studier (1983) interpreted the protected sites to be at or near 46.59, 63.19, and 92.40 (18 608, 25 224, and 36 900 bp, respectively). However, our interpretation of the Allet et al. results places the RNA polymerase binding site at 41.93 instead of at 92.40. One of the three different fragments we have isolated is an *Hpa*I fragment and appears to contain an intact promoter. The *Hin*fI$_{671}$ and *Ava*II$_{957}$ fragments each contain both the 36 827 site (what we believe to be the E promoter) and the 36 927 site. The major runoff transcript of each fragment comes from the 36 827 site. A very minor transcript may be initiating from the 36 927 site. This site may be a very weak promoter or what has been referred to as a tight binding site (Kadesch et al., 1980; West et al., 1980; Melancon et al., 1982). All three of the protected *Hpa*I sites are probably tight binding sites rather than promoters.

RNA fingerprint analysis and abortive initiation data together suggest that the E promoter transcript can initiate with either ATP or CTP, with ATP strongly favored. Heterogeneous initiation is not unprecedented, and although purine initiation is preferred, pyrimidine initiation has been observed [for a review, see Hawley & McClure (1983)].

Stahl & Chamberlin (1977) reported that the E promoter did not function transcriptionally at KCl concentrations greater than 100 mM. We observe maximal runoff transcription near 150 mM KCl (Figure 5). Although abortive initiation is inhibited greatly by increased [KCl], the E promoter still functions significantly at and above 150 mM. The high abortive initiation turnover rate at low salt may reflect a greater rate of dissociation of the abortive product from the initiation complex at low salt or conversely larger binding constants for the nucleotide substrates. It is possible that the high-salt maximum of runoff transcription is due to greater competency in completing the transcript.

The E promoter is characterized as a minor or weak promoter in comparison to the major A3 promoter. The kinetic constants and the predicted equilibrium constants reported here are consistent with these assignments. Isomerization from RP_i to RP_o at A3 ($t_{1/2} \approx 8$ s) is 2-fold faster than the rate of isomerization at the E promoter ($t_{1/2} \approx 16$ s). On the other hand, we find that the major promoter has at least a 10-fold stronger initial binding constant K_B. This is in agreement with our earlier report (Dayton et al., 1984) that two major promoters differed from the minor C and D promoters by a 10-fold larger value of K_B. For the T7 promoters, we believe K_B is the major factor in the promoter selection by RNAP in vitro and probably in vivo.

We have previously reported the kinetic parameters for A3 on a 493 bp *Hha*I fragment to be $k_2 = 0.04\ s^{-1}$ ($t_{1/2} \approx 17$ s) and $K_B \geq 4 \times 10^8\ M^{-1}$ (Dayton et al., 1984). This value of k_2 is a factor of 2 smaller than the value reported in this paper. The *Hha*I$_{493}$ fragment consists of DNA from −67 to +426 bp with respect to the A3 start site (+1), whereas the *Hpa–Hae*$_{810}$ fragment (used in this work) contains DNA from −99 to +711 bp. The additional DNA upstream of the −35 region on the *Hpa–Hae*$_{810}$ A3 promoter fragment might be sufficient to prevent interference from end effects such as RNAP end binding (Melancon et al., 1983) or breathing of the DNA at ends. However, we would have expected the proximity of an end to affect K_B, not k_2. A more likely explanation is that we reduced our experimental error for the A3 data by using CpA as the initiating nucleotide instead of ATP. The CpApU product is completely separated from the reactants by using the ascending paper chromatography system of Johnston & McClure (1976). We cannot rule out the possibility that base pairing of the CpA dinucleotide to the DNA might facilitate the melting of the DNA during isomerization of RP_i to RP_o and thus increase k_2.

Measurement of the approach to steady-state abortive initiation turnover adequately determines the parameters k_2 and K_B for weak promoters such as the E promoter, but for very strong promoters, the K_B determination is only an estimate, a lower limit to the actual value. The graphical representation of the nonlinear analysis for E (Figure 6B) has relatively minor scattering of the data about the fitted curve and has data on either side of [RNAP] = $1/K_B$. Valid calculations of K_B require data on both sides of $1/K_B$ (Currie, 1982). The nonlinear plot of the A3 data has relatively large scattering of the data and no data near $1/K_B$ (Figure 7). For the latter reason, we cannot easily derive the curvature of the line from

the data. The concentration of unbound RNAP must remain essentially constant for the equations of McClure (1980) to be valid. Experimentally, promoter concentration must be ≥1 nM for most promoters in order to obtain enough product per unit time for valid determination of k_{obsd} (eq 1). Therefore, 10 nM RNAP is the minimum [RNAP] which should be used. The maximum K_B which can be reliably measured is thus about 1×10^8 M^{-1} using this experimental technique. There may be some difficulty with very weak promoters if K_B is $<1 \times 10^6$ M^{-1}, because use of very high polymerase concentrations often results in aberrant data, perhaps because of interference from nonspecific binding. In general, when $k_2 > 0.1$ s^{-1} ($1/k_2 < 10$ s), estimation of k_2 by this method is also tenuous. Thus, typical experimental conditions limit determination of statistically useful data to promoters with K_B values between 1×10^6 and 1×10^8 M^{-1} and k_2 less than 0.1 s^{-1}.

Wiggs et al. (1979) reported that the half-life of dissociation for the E promoter measured with heparin as the sequestering agent was much less than 5 min. We found the dissociation of the E promoter–RNAP complex to be more than 15-fold faster when challenged with heparin ($t_{1/2}$ = 0.9 min) than when challenged with poly[d(A-T)] ($t_{1/2}$ = 13 min). The A1 promoter has also been shown to be unusually heparin sensitive (Stahl & Chamberlin, 1977; Wiggs et al., 1979; Dayton et al., 1984). It has been reported that the A2, A3, and D promoters (but not A1) could be challenged equivalently by either heparin or a copolymer (Cech & McClure, 1980; Dayton et al., 1984). An interesting observation is that RNA polymerase at both A1 and E is sensitive to heparin attack and utilizes the nucleotide monophosphate AMP at a substantially faster abortive initiation rate than the dinucleotide monophosphate CpA. Conversely, RNA polymerase at A3 is not very sensitive to heparin attack and utilizes CpA at a faster abortive initiation rate than AMP. Thus, there may be a correlation between heparin sensitivity of RNAP–promoter complexes and the preference for abortive initiation substrates.

Although we do not understand the mechanistic basis of selective heparin sensitivity, heparin clearly perturbs kinetic measurements for some promoters. Methods that employ the addition of heparin at the same time nucleotide triphosphates are added to initiate synthesis of one round of transcription (Stefano & Gralla, 1980, 1982) may give results that are difficult to interpret if the polymerase–promoter complex is destabilized before σ is released and elongation proceeds.

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Registry No. RNA polymerase, 9014-24-8.

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Covalent Affinity Labeling, Detergent Solubilization, and Fluid-Phase Characterization of the Rabbit Neutrophil Formyl Peptide Chemotaxis Receptor†

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ABSTRACT: The formyl peptide chemotaxis receptor of rabbit neutrophils and purified rabbit neutrophil plasma membranes has been identified by several affinity labeling techniques: (1) covalent affinity cross-linking of *N*-formyl-Nle-Leu-Phe-Nle-^{125}I-Tyr-Lys (^{125}I-hexapeptide) to the membrane-bound receptor with either dimethyl suberimidate or ethylene glycol bis(succinimidyl succinate) and (2) photoactivation of *N*-formyl-Nle-Leu-Phe-Nle-^{125}I-Tyr-N^{ϵ}-[6-[(4-azido-2-nitrophenyl)amino]hexanoyl]Lys (^{125}I-PAL). These techniques specifically identify the receptor as a polypeptide that migrates as a broad band on sodium dodecyl sulfate–polyacrylamide electrophoresis, with M_r 50 000–65 000. The receptor has been solubilized in active form from rabbit neutrophil membranes with the detergents 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and digitonin and from whole cells with CHAPS. Chemotaxis receptor activity was measured by the ability of the solubilized membrane material to bind ^{125}I-hexapeptide or fMet-Leu-[^{3}H]Phe with gel filtration or rapid filtration through poly(ethylenimine)- (PEI) treated filters as assay systems. ^{125}I-PAL was specifically cross-linked to the same molecular weight material in the CHAPS and digitonin solubilized extract, but no specific labeling of the receptor was seen when membranes were extracted with Nonidet P-40 and Triton X-100. Therefore, although a large number of detergents are able to solubilize the receptor, it appears that some release the receptor in an inactive form. The ligand binding characteristics of fMet-Leu-[^{3}H]Phe to the CHAPS-solubilized receptor shared properties with the membrane-bound formyl peptide receptor, both of which showed curvilinear, concave-upward Scatchard plots. Computer curve fitting with NONLIN and statistical analyses of the binding data indicated that for both the membrane-bound and solubilized receptors a two saturable sites model fitted the data significantly better ($p < 0.01$) than did a one saturable site model. The characteristics of the two saturable sites model for the soluble receptor were a high-affinity site with a K_D value of 1.25 ± 0.45 nM and a low-affinity site with a K_D value of 19.77 ± 3.28 nM. A total of 35% of the two sites detected was of the higher affinity. In addition, a Hill coefficient of 0.61 ± 0.12 was observed.

Structural identification and detergent solubilization of specific membrane receptors is required to understand ligand–receptor interactions and the subsequent activation pathways that result in a multitude of effector responses. Since the initial discovery of the formyl peptide receptors (Showell et al., 1976; Aswanikumar et al., 1977; Williams et al., 1977) and the numerous biological responses that are mediated through them [reviewed in Becker (1979), Snyderman & Goetzl (1981), and Becker & Marasco (1985)], structural information has lagged behind the advances made in our understanding of the activation pathways and other functional properties of the receptor. Only recently has limited structural information been obtained. Niedel et al. (1980) first showed that the receptor can be covalently affinity labeled and also extracted from the neutrophil in a soluble active form (Niedel, 1980), findings that have been abundantly confirmed (Dolmatch & Niedel, 1983; Schmitt et al., 1983; Baldwin et al., 1983). The receptor on the human neutrophil is a glycoprotein

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